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ENDOMETRIAL REGENERATION: UNRAVELING THE MYSTERIES OF THE STROMA

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Endometrial Regeneration: Unraveling the Mysteries of the Stroma

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Dedicated to my family

ABSTRACT

The healthy endometrium has the unique capacity of shedding and regenerating its functional layer once every menstrual cycle, under the regulation of hormones, progenitor cell populations and inflammatory mediators. Thanks to this meticulously timed cycle the uterus is regularly renewed in preparation for blastocyst implantation. However, if any of these factors are perturbed, the result may be endometrial disorders with intrauterine scar formation, dysregulated proliferation and heavy menstrual bleeding. This PhD project addresses the interplay of these factors specifically within the endometrial stromal compartment in ensuring healthy endometrial regeneration and homeostasis, thereby investigating future therapeutic targets for benign gynecological disorders. Cells and tissue from endometrial biopsies and bone marrow aspirate were studied using multiple cellular and molecular techniques. Stromal cells have been characterized in terms of their phenotype, their transcriptome and their ability to immunomodulate, providing a starting point for future endometrial stromal cell therapy development.

In **study I**, the transcriptional profile of progesterone receptor modulator associated endometrial changes (PAEC) was studied in women with three months of continuous mifepristone treatment to understand the future implications of PAEC and the safety of long-term mifepristone use. Our microarray findings indicate that progesterone withdrawal and unopposed estrogen surge alter the endometrial structural organization and extracellular matrix composition, particularly affecting the stromal compartment in the tissue. No differentially regulated genes were involved in endometrial-cancer associated pathways.

In **study II**, endometrial stromal cells (eSCs) were characterized in terms of their phenotype, immunomodulation and tumorigenicity for early pre-clinical cell therapy development. eSCs demonstrated a mesenchymal stromal cell (MSC) surface marker profile and multipotency, while retaining chromosomal stability and showing no tumorigenicity after *in vitro* expansion. When stimulated with pro-inflammatory cytokines, eSCs presented with an anti-inflammatory phenotype and secreted immunomodulatory factors, but did not express human leukocyte antigen class II on their cell surface. eSCs suppressed CD4+ T cell proliferation and activation while significantly modulating their differentiation state, upregulating CD4+ effector memory T cells.

The cellular diversity within the endometrial functional layer's stromal compartment was explored in **study III** using single cell RNA sequencing. Different computational tools and an external dataset were used for analysis and validation. The study revealed diverse stromal subsets with transcriptional profiles representing different stromal activation states and niches important in wound healing, regeneration and immunomodulation. Several of these stromal transcriptional profiles could be validated in placenta derived decidua, suggesting the transcriptional profiles can withstand cycle changes and placentation.

In **study IV** we determined the effects of blood exposure on bone marrow (BM) MSC viability and immunomodulatory functions, exposing BM MSCs to human blood products *in vitro* and evaluating their interactions with complement and the peripheral immune repertoire. Plasma exposure induced lysis of BM MSCs, while surviving BM MSCs had C3c bound to their surface. The MSC secretome reduced monocyte number and recruitment with many remaining monocytes skewed towards a classical, anti-inflammatory phenotype. Frequencies of immune modulating myeloid derived suppressor cells (MDSCs), both monocytic and polymorphonuclear, were also increased in response to BM MSCs. These data indicate that MSCs rapidly die once exposed to blood, but can still exert an anti-inflammatory response through the skewing of monocytes and upregulation of MDSCs. We hypothesize that this shift in the peripheral repertoire indirectly regulates adaptive immune cells' response to the long-term tolerogenic effect seen after MSC intravenous infusion.

In summary, endometrial regeneration is tightly regulated by hormones, immune cells and their interactions with stromal cells. Our research has provided new, detailed information on the stromal compartment by demonstrating there are multiple stromal subsets with different transcriptional profiles, presumably fulfilling multiple roles. Furthermore, eSCs have the ability to respond to inflammatory cues, as well as directly and indirectly modulating immune cells through their secretome. Additionally, BM MSCs exposed to blood can modulate the innate immune response even after cell death due to their release of soluble factors which induce an anti-inflammatory monocyte phenotype which lasts beyond their clearance from circulation. We conclude that stromal cells from different tissues have exceptional regulatory capacities in tissue homeostasis, inflammation and healing.

LIST OF SCIENTIFIC PAPERS

I. Molecular Characterization of PRM-Associated Endometrial Changes, PAEC, following Mifepristone Treatment
Berger C, Boggavarapu N, Norlin E, **Queckbörner S**, Hörnaeus K, Falk A, Engman M, Ramström M, Lalitkumar PGL, Gemzell-Danielsson K
Contraception. 2018;98(4):317-22.

II. Endometrial Stromal Cells Exhibit a Distinct Phenotypic and Immunomodulatory Profile
Queckbörner S, Syk Lundberg E, Gemzell-Danielsson K, Davies LC
Stem cell research & therapy. 2020;11(1):1-15.

III. Stromal Heterogeneity in the Proliferative Endometrial Functionalis - A Single-Cell Approach
Queckbörner S, von Grothusen C, Boggavarapu N, Davies LC, Gemzell-Danielsson K
Manuscript

IV. Lysis of Mesenchymal Stromal Cells Upon Blood Contact Triggers Anti-inflammatory Skewing of the Peripheral Immune Repertoire
Davies LC, **Queckbörner S**, Ström C, Törnqvist Andrén A, Plym Forshell T, Le Blanc K
Manuscript Submitted

CONTENTS

LIST OF ABBREVIATIONS

AS	Asherman's Syndrome
ATMP	Advanced Therapy Medicinal Product
BM MSC	Bone Marrow Derived Mesenchymal Stromal Cells
BMMNC	Bone Marrow Mononuclear Cells
C3	Complement Component 3
CD	Cycle Day
CFU-F	Colony Forming Units-Fibroblast
CM	Central Memory
E2	17β-estradiol
ECM	Extracellular Matrix
EEP	Endometrial Epithelial Progenitor
EM	Effector Memory
eSC	Endometrial stromal cells
ESP	Endometrial Stromal Progenitor
eVs	Extracellular vesicles
FAS	Fas Cell Surface Death
GEM	Gel bead-in-Emulsion
GvHD	Graft-versus-Host-Disease
HI	Heat Inactivated
HLA	Human Leukocyte Antigen
HMB	Heavy Menstrual Bleeding
IBMIR	Instant Blood Mediated Inflammatory Reaction
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon Gamma
ISCT	International Society for Cell & Gene Therapy
IV	Intravenous
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
M1	Macrophages Subtype 1
M2	Macrophages Subtype 2
MAST	Model-based Analysis of Single-Cell Transcriptomics test
MCP-1	Monocyte Chemoattractant Protein-1
MDSC	Myeloid-Derived Suppressor Cells
menSCs	Menstrual Blood-derived Stromal Cells
MHC	Major Histocompatibility Complex
MMP	Matrixmetalloprotease
MOA	Mode of Action
MSC	Mesenchymal Stromal Cell
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NK	Natural Killer
P4	Progesterone
PAEC	Progesterone Receptor Modulator Associated Endometrial Changes

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCA	Principal Components Analysis
PDGFRß	Platelet-Derived Growth Factor Receptor ß
PG	Prostaglandin
PR	Progesterone Receptor
PRM	Progesterone Receptor Modulator
RIF	Repeated Implantation Failure
SAM	Significance Analysis of Microarrays
scRNA-seq	Single cell RNA Sequencing
SOX9	Sry-box Transcription Factor 9
TCR	T Cell Receptor
TF	Tissue Factor
TGFβ1	Transforming Growth Factor Beta 1
TLR	Toll-like Receptors
TNFα	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION

Endometrial regeneration, spontaneous decidualization and menstruation are a unique evolutionary adaptation which humans share with few other mammals, specifically apes, monkeys, elephant shrews and select bats (1). The endometrium undergoes approximately 450 regenerations in a woman's reproductive lifetime demonstrating a timely, hormonally controlled tissue turnover not observed in any other system (2). Despite this, little attention has been given to the endometrium either by the field of gynecology, or by the greater scientific community, as an *in vivo* model for inflammation and wound healing. While the underlying reasons for this are unclear, it is likely influenced by a combination of societal factors, which ultimately result in research concerning women's benign gynecological disorders being overlooked and underfunded. In a similar thread, research on the endometrium has more frequently focused on the role of the endometrium in the context of embryo implantation, prioritizing the decidualization and placentation processes over regeneration (3-5). Although much progress has been made in recent decades, with the integration of high throughput molecular techniques in the field, many questions remain unanswered concerning human endometrial tissue complexity, and the mechanisms controlling menstruation and endometrial regeneration. More basic research is needed to explain the role of immune, stromal, epithelial and vascular cell crosstalk in regulating endometrial regeneration. To fully grasp benign endometrial disorders such as Asherman's syndrome and heavy menstrual bleeding, an understanding of healthy tissue homeostasis at the beginning of the cycle is required.

My PhD aims to provide a clearer understanding of the cellular composition in the proliferative phase endometrium. I have sought to determine the cell diversity, lineage, cell surface expression and molecular expression of different cell types within the stromal compartment. Emphasis has been placed on stromal subsets from the endometrial *functionalis,* their potential as a novel cell source for cellular therapy and their role in endometrial tissue homeostasis and pathophysiology. I have also taken a broader approach to cell therapy, specifically looking into bone marrow mesenchymal stromal cell mode of action upon systemic delivery.

1.1 ENDOMETRIAL MORPHOLOGY

The mucous lining of the uterus is collectively referred to as the endometrium and is composed of two layers: the *basalis* and the *functionalis*. The *functionalis* is the superficial layer where growth, deterioration and shedding is hormonally regulated by the hypothalamic-pituitary-ovarian axis during the menstrual cycle (on average between 21-35 days) (6). Following menstruation, the *functionalis* regenerates from the *basalis*. Commonly this growth cycle is divided into the following stages: menstrual (standardized cycle day (CD) 1-4), proliferative (CD 5-14) and secretory (CD 14-28) (5). Established cell populations within the endometrium include luminal epithelial, glandular epithelial, stromal, vascular (endothelial, smooth muscle, mural) and leukocytes (monocytes, primarily macrophages subtype 2 [M2], dendritic cells and natural killer [NK] cells, eosinophils, neutrophils, T cells, B cells and mast cells) (7, 8). The stromal compartment makes up the largest portion of the tissue and is covered by luminal epithelium. Tubular glands reach up from the *basalis* to the surface of the *functionalis* and are lined by glandular epithelium. Spiral arteries ensure blood supply to the tissue (9). The leukocytes are predominantly found in the stromal compartment and vary in distribution and abundance throughout the hormonal cycle with varying degrees of activation (10). The endometrium is a tissue which combines autocrine signaling, dynamic leukocytes and a highly responsive somatic cell population to ensure a meticulously timed menstrual cycle.

1.2 HORMONAL REGULATION OF THE MENSTRUAL CYCLE

The menstrual cycle runs parallel to the ovarian cycle and can be divided into three phases: the proliferative phase, which corresponds to the ovarian follicular phase; the secretory phase, which corresponds to the ovarian luteal phase; and the menstrual phase (depicted in **Figure 1**). In the proliferative phase, primary follicles in the ovary start producing 17β-estradiol (E2) leading to the proliferation of epithelial, vascular and stromal cells, while new extracellular matrix (ECM) is laid down resulting in thickening of the endometrial tissue. Ovulation occurs midcycle, approximately CD 14, which is followed by the secretory phase. During the secretory phase, under the influence of pregn-4-ene-3,20-dione (progesterone; P4) produced in the *corpus luteum,* the endometrium undergoes functional differentiation with glands taking on a tortuous shape and increasing their secretion of glycoproteins (11, 12). P4 peaks in the mid-secretory phase leading to stromal decidualization; meaning transformation of

stromal cells from an elongated fibroblast morphology to a larger polygonal epithelioid shape. These changes are modulated by decidualization/ progesterone-dependent proteins and polysaccharides including: prolactin, glycogen, tissue factor (TF), insulin-like growth factor-binding protein 1, forkhead box o1 and cyclic adenosine monophosphate (12-15). In the absence of fertilization and embryo implantation the *corpus luteum* regresses and P4 levels drop (6). While the hormone levels decrease, the endometrium sheds itself with enhanced apoptosis within the epithelial compartment (16). At the same time there is an increase of all leukocytes just before menstruation, which are reported to constitute 40% of all cells in the stroma (10, 17, 18). All cell populations in the endometrium are responsive to hormonal cues, yet it is within the stromal compartment that the greatest changes in cell cycle, cell function and morphology are observed. Aside from hormonal regulation, there are other mechanisms in place e.g. wound healing, which enable successful initiation of continued cycling.

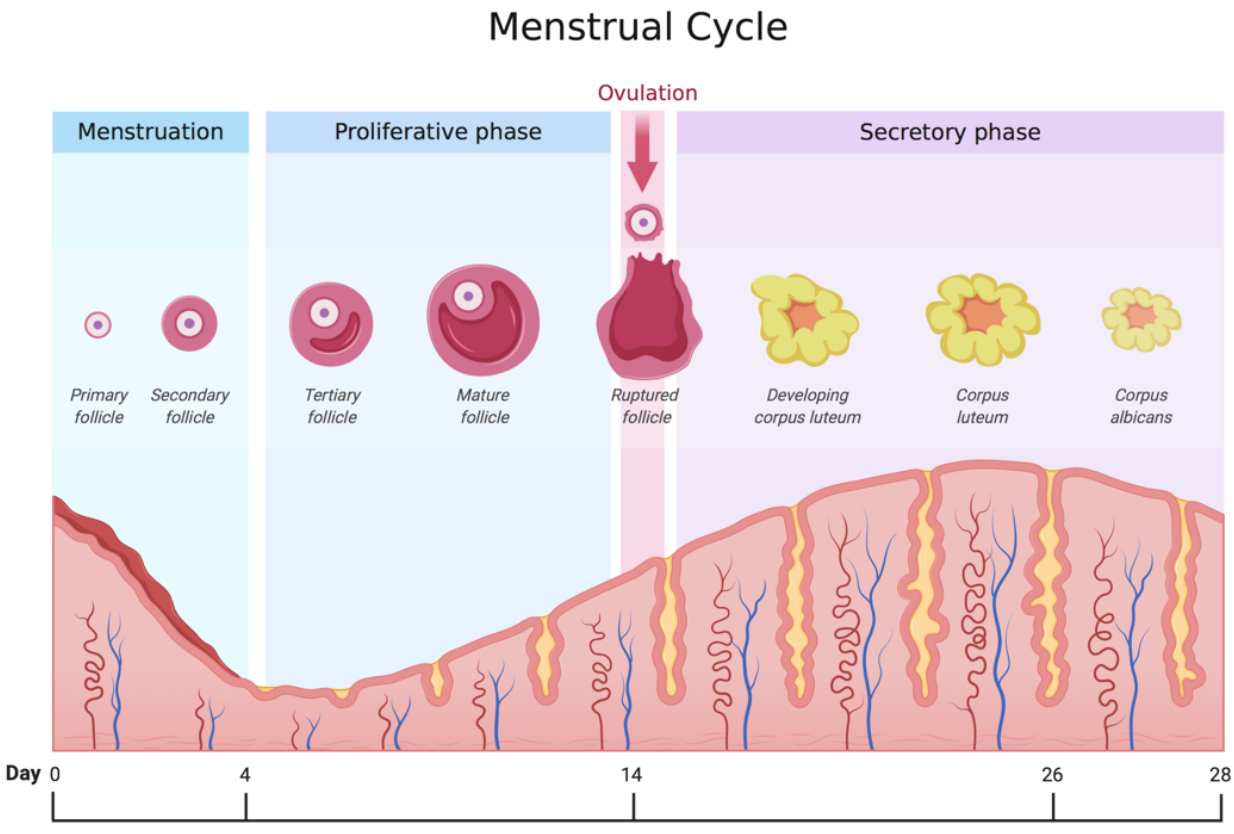

Figure 1: Human menstrual cycle
Schematic diagram of the endometrium during the menstrual cycle, illustrating the physiological changes seen in menstruation, proliferative phase and secretory phase under estrogen and progesterone control. Figure reproduced with permission from Biorender.

1.3 THE INFLAMMATORY EVENTS OF ENDOMETRIAL BREAKDOWN AND REPAIR

Inflammation is an unspecific response by the immune system to a foreign stimulus which eliminates infection or repairs injured tissue to restore homeostasis (12). The menstrual cycle can be classified as a series of contained inflammatory events spanning from tissue breakdown and repair in menstruation/ regeneration to tolerance/ rejection in decidualization and implantation (19). Hormonal and inflammatory regulation of the menstrual cycle are tightly interwoven (19), however the precise cascade of events controlling menstruation and repair is still an active field of research.

Menstruation is initiated by the withdrawal of P4 from the endometrial vasculature and perivascular stroma by indirect activation of nuclear factor kappa B (NF-κB) pathway at the end of the secretory phase (20). Inflammatory events including the release of chemokines, cytokines, growth factors, lipid mediators, complement and prostanoids (prostaglandins and prostacyclins) orchestrate the activation and migration of immune cells to the endometrium (12). Simultaneously, stromal cell adhesion properties are altered and an increase in vascular permeability and vasodilation in the spiral arterioles enables endometrial shedding and vascular remodeling. Neutrophils are amongst the first immune cells recruited via the spiral arterioles. Tissue breakdown is led by focal activation of proteolytic matrix metalloproteases (MMPs) degrading ECM components and basement membrane (20). Depending on the microenvironment different neutrophil granules are released e.g. MMP9, proteinase 3 and elastase (20). These changes activate latent pro-inflammatory mediators including tumor necrosis factor alpha (TNFα), interleukin (IL)1β and recruit monocytes to the site of injury, polarizing them towards an macrophages subtype 1 (M1) phenotype (20).

Towards the end of menstruation, the combined clotting system of platelet aggregation, fibrin deposits and thrombi formation begin hemostasis and damaged blood vessels contract (21). The subsequent release of growth factors and TF initiate wound healing and angiogenesis (21). Anti-inflammatory lipoxins contribute to the neutrophil clearance by inducing apoptosis. This is accompanied by phagocytosis of tissue debris and apoptotic neutrophils. Macrophages sense apoptotic cells in their environment adjusting their phenotype to M2 and releasing anti-inflammatory IL10, transforming growth factor beta 1 (TGFβ1) and further lipid mediators(12)(see **Figure 2**).

Menstruation can also be pharmaceutically induced by the administration of a progesterone agonist e.g mifepristone delivered during the mid-luteal phase. In animal models, this provides a suitable model to study inflammatory events in spontaneous menstruation (22). Although the menstrual cycle is frequently viewed as a series of consecutive steps, menstrual and proliferative phases are not absolute as tissue breakdown and regeneration occur in parallel at different foci (23). Thus, inflammatory mediators involved in tissue breakdown are also important regulators in ensuing tissue repair and data from these stages should be considered collectively.

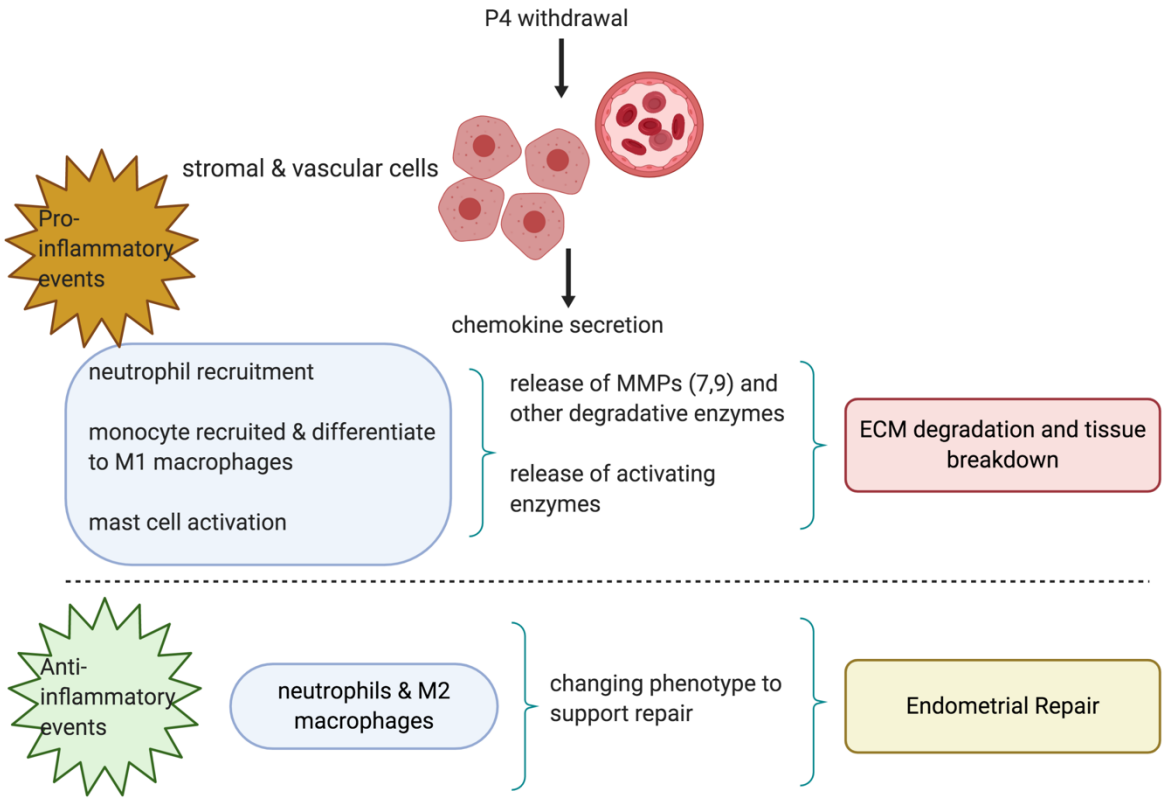

Figure 2: Inflammatory events in menstruation and regeneration
Schematic diagram of inflammatory mediators and immune cells involved in endometrium breakdown and repair. Abbreviations: (P4) Progesterone; (ECM) extracellular matrix, (MMP) matrix metalloproteases. Diagram was created with BioRender adapted from *Evans and Salamonsen. Rev Endocr Metab Disord, (2012) 13:277–288.*

1.3.1 INFLAMMATORY MEDIATORS

Several inflammatory mediators are essential for inflammation regulation in tissue breakdown and repair and contribute to the feedback loop controlling the menstrual cycle. Prostaglandin (PG) is an important mediator in inflammation and wound healing. In the endometrium, P4 inhibits PG synthesis by the suppression of cyclooxygenase 2 (COX2) and

induction of PG dehydrogenase. When P4 levels drop during the late secretory phase, COX2 levels rise in stromal, epithelial and perivascular cells with increased PG synthesis (20). PGs contribute to vasodilation and smooth muscle contractions which aids in the shedding of decidualized tissue at menstruation (12). Furthermore, PGE2 increases IL6 and IL8 expression via PGE2 and PGE4 receptor indirectly contributing to leukocyte recruitment and edema formation (24). With respect to tissue repair, PGE2 increases the expression of growth factors specifically involved in angiogenesis, such as adrenomedullin, vascular endothelial growth factor (VEGF) and connective tissue growth factor (12). Lipid and protein mediators have been identified as possible contributors to tissue repair due to their ability to inhibit leukocyte function and pro-inflammatory cytokine secretion (25). Hofer et al. identified lipoxins as amongst the most abundant metabolites of arachidonic acid breakdown in menstrual blood. Arachidonic acid is a precursor to PGs (26).

Pro-inflammatory cytokines associated with menstruation and orchestrating the initial tissue breakdown include TNFα, interferon gamma (IFNγ) and IL1β. Parallel to the increase in apoptosis seen towards the end of the secretory phase and during menstruation TNFα levels progressively increase during the secretory phase and reach a maximum concentration at menstruation (27, 28). IL6 is a cytokine produced by stromal cells, endothelial cells and immune cells. IL6 is produced in damaged/ activate endometrial stromal cells (eSCs) via toll-like receptors (TLR) signaling and is important for subsequent immune cell recruitment and later tissue homeostasis. There is no conclusive data on whether IL6 is regulated by E2 or P4 in eSCs however IL6 secretion is dose dependently stimulated by IL1β and the combined expression of IFNγ and TNFα (29). Thus, IL6 contributes to menstrual shedding directly or by modulating other cytokines and immune cells (30). IL10 is an anti-inflammatory cytokine secreted by macrophages to encourage wound healing, lower concentrations of systemic and tissue level IL10 have been measured in several benign gynecological disorders compared to healthy controls (31, 32).

1.3.2 MONOCYTES AND MACROPHAGES

Monocytes make up 10% of all circulating leukocytes and are recruited to an injury site during inflammation (33). As the precursors to macrophages and dendritic cells their cell fate is central to controlling inflammation. They can generally be classified into three subsets based on their cell surface markers: classical/ inflammatory (CD14+CD16-), non-

classical/anti-inflammatory (CD14-CD16+) and the intermediate subset (CD14+CD16+ HLA II++) (33). Macrophages respond to environmental stimuli produced during injury or infection as well as adaptive immune cells. Macrophages can be broadly classified as pro-inflammatory (M1) and anti-inflammatory (M2). M1 have anti-microbial activity, they secrete IFNγ and TNFα contributing to inflammation. M2 contribute to tissue repair and remodeling by secreting TGFβ1 and IL10 (33).

Monocytes and macrophages are found at all stages of the menstrual cycle; however, an increase is seen prior to menstruation and during the early proliferative phase (20, 34, 35). Macrophages take part in phagocytic clearance of apoptotic cells during menstruation and are part of the tissue remodeling process during the proliferative phase, responding to E2 and glucocorticoids due to their cell surface expression of estrogen receptor beta and glucocorticoid receptor (GR) (12, 36, 37). Once macrophages have recognized peripheral phagocytosed apoptotic cells they change to an anti-inflammatory phenotype with the secretion of IL10, TGFβ, lipoxins and resolvins (38, 39). In a study looking at the correlation between cellular apoptosis and number of macrophages (CD68+) in human endometrium, the greatest number of macrophages and apoptosis was seen in the late secretory and early proliferative phases in line with the time when the tissue undergoes considerable tissue breakdown and repair (34). Less is known about the role of monocytes in endometrial repair, however higher expression of CD71, CD69 and CD54 on monocyte cell surface have been observed at this stage of the cycle. These markers are associated with proliferation, adhesion and activation suggesting their role in regulating and modulating the immune response(40). Furthermore, recent work has suggested monocytes are cleared by apoptosis once endometrial repair has been completed (41). Cousins et al. also identified several distinct monocyte/ macrophage populations which were putatively classified as classical monocytes, monocyte-derived macrophages and tissue-resident macrophages which all localized to areas of tissue breakdown and repair in the endometrium *functionalis* (41).

M2-like activities are more abundant during the proliferative phase, in line with wound healing, remodeling and proliferation (42). In the context of endometriosis, M2 are most prevalent in proliferative endometriotic lesions. An *in vivo* study in mice has shown that M1 obstruct lesion progression while M2 contribute to elevated unwanted endometrial stromal and epithelial proliferation. In another study, eutopic and ectopic endometrial stromal homogenates induced immunological tolerance of monocyte-derived macrophages *in vitro*,

polarizing the cells from M1 towards a M2 phenotype following co-culture. By neutralizing IL6 M2 polarization could be reversed(43).

1.3.3 T CELLS

T cell are a central component of the adaptive immune system and exert their action by cell mediated immunity; modulating other immune cells, endothelial cells and parenchymal cells (44). T cells are divided into two classes CD4+ T cells (T helper cells) which can activate macrophages and CD8+ T cells (cytotoxic T cells) which identify and kill infected cells. Via the T cell receptor (TCR), T cells can recognize antigen bound to the major histocompatibility complex (MHC). CD8+T cells recognize their cognate antigen presented by major histocompatibility complex (MHC) class I on antigen-presenting cells while MHC class II presents the cognate antigen to CD4+ T cells (45). Regulatory T cells (Tregs) are a T cell subset which counter acts T-cell mediated responses, with an overall immunosuppressive effect (44). Memory T cells are antigen-specific T cells which ensure the long-term recall of antigens and provide a rapid response on re-exposure, they are classified as long-lived circulating central memory (CM) T cell or tissue resident effector memory (EM) T cell (46, 47).

T cells are present throughout the menstrual cycle, however relative to other lymphocytes, they are considerably fewer in number (48). T cells can be found sparsely distributed in the epithelium and stroma, with lymphoid aggregates forming with B cells specifically during the secretory phase (49). Furthermore, unlike in peripheral blood, the ratio of CD4+ T cells and CD8+ T cells is inverted with more CD8+ T cells than CD4+ T cells. This is an interesting adaptation which hasn't been investigated further in endometrium with regards to regeneration/ fibrosis. Presumably, this is a sign of increased immune activation indicative of changes in T cell migration and differentiation or an accumulation of CD8+ T cells in tissue (50). Interestingly, this is also seen in the mucosal lining of the intestine and has previously been explored in the context of liver fibrosis (51-53). The cytolytic potential of CD8+ T cells has been shown to be highest in proliferative phase endometrium and in endometrium from postmenopausal women, suggesting they are regulated by P4 and E2 (51). Although not specifically investigated within the proliferative phase endometrium, several human studies have demonstrated that the majority of CD4+ T cells and CD8+ T cells in the endometrium are activated (CCR5+) EM, a phenomenon also seen in placenta decidua (45,

52, 54). Particularly the CD8+ EM T phenotype has been investigated and discussed in the context of pregnancy, fetal tolerance and infection however what this means for endometrial regeneration still needs to be determined.

With regards to Tregs, there is conflicting data concerning their abundance and regulation during the menstrual cycle, with some research suggesting they are more abundant during menstruation while others say during proliferative phase (55, 56). This is partially due to the disparities in the cell surface markers used to characterize Tregs and the difficulty to distinguish between the two phases precisely (56). Considerably more research has investigated the role of Tregs in endometriosis, suggesting increased Tregs in endometriotic lesions compared to ectopic endometrium and eutopic endometrium (56, 57).

1.4 ENDOMETRIAL REGENERATION AND THE PROGENITOR CELL NICHES

Within 48 hours of menstruation, re-epithelialization of the injured tissue is initiated. Remaining luminal epithelium surface regions and epithelial glandular crypts heal, resealing the denuded stroma to re-establish mucosal homeostasis (23). As in other outward-facing mucosal linings, like the intestinal epithelium, the luminal epithelium provides an external barrier to invading pathogens (58). At menses the endometrium is thin (2-4 mm) with narrow, short glands. During the proliferative phase, the glands elongate and take on a coiled structure with interspersed stroma expanding simultaneously (5-11 mm) (23). Unquestionably, there is a close interaction between the stromal and epithelial cells during proliferation. Epithelial signals direct the hormonal stimulation of the stroma, thereby ensuring paracrine control of tissue growth (59). However, in the absence of hormonal support (oophorectomized) the endometrium is also able to heal itself without scarring, suggesting normal wound healing can override hormonal modulation to guarantee tissue repair when needed (60).

It is generally accepted that the endometrium has a pool of progenitor cells that regulate its cyclical regeneration into epithelial, stromal and endothelial cells. This premise was first proposed by Chan et al. in 2004 when magnetic bead-selected epithelial cells (BEREP4+CD45-) and stromal cells (BEREP4-CD45-) were grown at single cell density on fibronectin and a fibroblast feeder layer. By fulfilling the criteria of achieving colonies of varying sizes in both populations and their adhesion to plastic they proposed the existence of

putative endometrial progenitors (61). They hypothesized that approximately 0.22–0.52% of total epithelial and 1.25% of total stromal cells possess exceptional clonogenic potential *in vitro*. At this point, initial comparisons were made to mesenchymal stromal cells (MSCs) in other regenerative tissues (**See Section 1.6 Mesenchymal Stromal Cells**) (61). Following the release of the International Society for Cell and Gene Therapy's (ISCT) MSC characterization guidelines, distinctions were made between the endometrial epithelial progenitor (EEP) seen in luminal and glandular epithelium and the stromal progenitor (ESP) found in the perivascular environment of the stromal compartment (62) (**see Figure 3**). For clarification purposes, unspecific endometrial stromal cells (bulk) will be referred to as eSCs from this point forward to distinguish between specific subsets when needed.

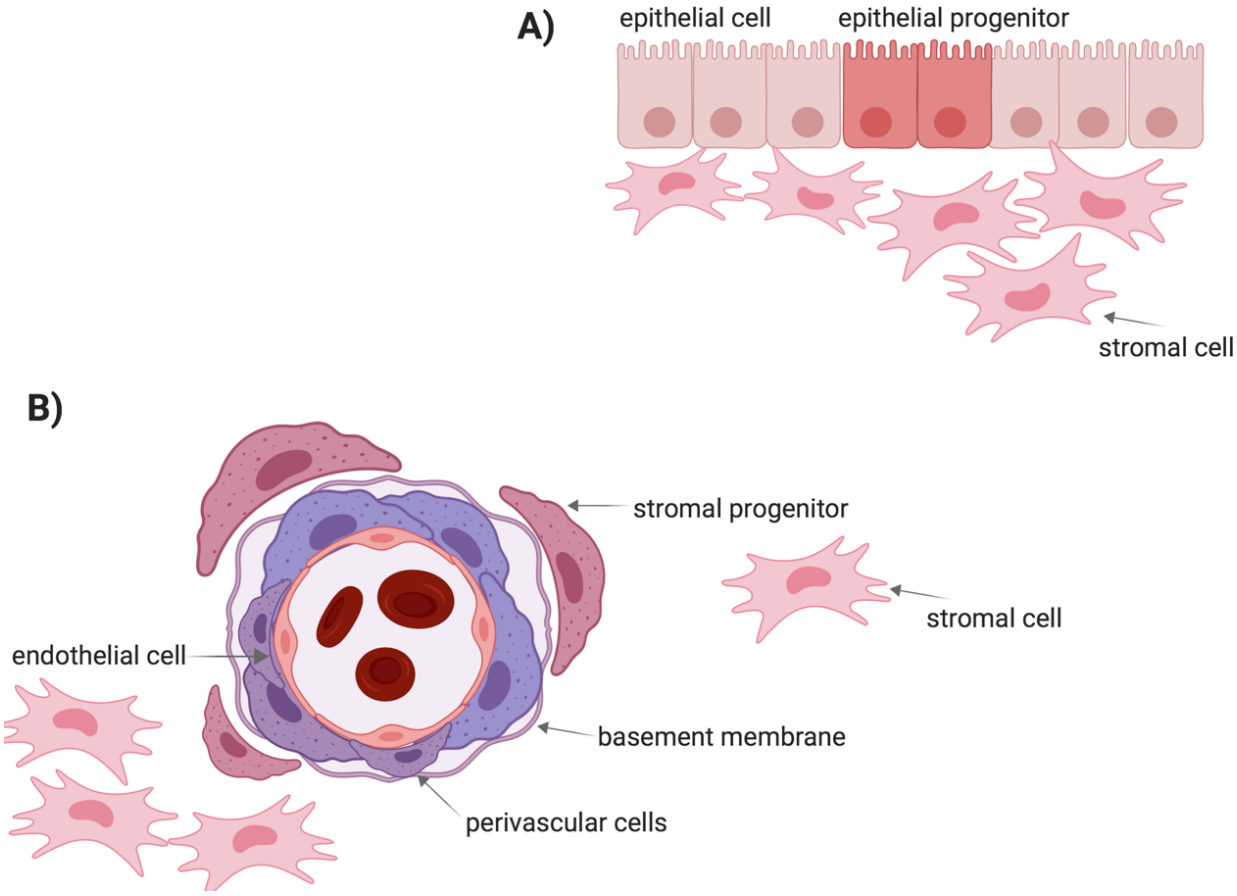

Figure 3: Endometrial progenitor environments involved in tissue regeneration
Two thirds of the endometrium make up the *functionalis* and one third the *basalis*. Glandular epithelium extends from the base of the *basalis* to the top of the *functionalis* where the luminal epithelium covers the stroma. **A** The endometrial epithelial niche, containing epithelial progenitor cells (*dark pink*) and epithelial cells contributing to re-epithelization. Within the stromal compartment the vasculature is surrounded by endothelial cells and perivascular cell (mural cells and smooth muscle cells) and endometrial progenitor stromal progenitor cells, this constitutes the B Endometrial perivascular environment. The diagram was created with BioRender.

1.4.1 PERIVASCULAR ENVIRONMENT

Early work aimed at drawing comparisons between ESPs and MSCs. Expanded ESPs presented the same cell surface expression proposed by the ISCT for MSCs: positive for CD90+, CD73+, CD105+ (> 95%) and negative for CD45-, CD34-, CD14-, CD19- and human leukocyte antigen class II (HLA class II; < 2%) (63). However, these surface markers are equally expressed on fibroblasts while CD73 has been shown to be upregulated on the stromal cell surface once expanded on plastic *in vitro* and as such may be a marker enhanced by these culture conditions (64). Consequently, it is difficult to distinguish ESPs from the greater eSCs on the basis of cell surface expression alone (65). Furthermore, it can be assumed that long-term expansion of progenitor cells outside of their *in vivo* niche, may lead to their terminal differentiation. To establish a hierarchy within the stromal compartment i.e. to distinguish between fibroblasts, transit amplifying cells and progenitor cells more specific markers are required.

The current commonly applied marker combination is CD146+ and platelet-derived growth factor receptor ß (PDGFRß+), which identifies a perivascular population *in vivo* (63, 66, 67). It has been proposed that *in vitro* expanded or fresh flow cytometry sorted stromal cells expressing CD146+ PDGFRß+ identifies ESPs, CD146- PDGFRß+ fibroblasts and CD146+ PDGFRß- endothelial cells (67). As a follow up, Spitzer et al. performed a microarray analysis comparing gene expression between fibroblasts and ESPs, revealing differentially regulated genes associated with Notch1, TGFß1, Insulin-like growth factor 1, sonic hedgehog, and G-protein-coupled receptor, encouraging the premise of two distinct stromal profiles. Sushi domain containing 2 (SUSD2) has been proposed as a single marker which can replace the CD146+ PDGFRß+ marker combination for ESPs (68). Although these markers have been able to distinguish a perivascular cell, there have been very few functional studies with rodents or human biopsies showing capacity for ESPs to differentiate down multiple endometrial lineages, and the ability to long-term self-renew (69). Ultimate proof of ESP multipotency would be their ability to produce a vascularized stroma *in vivo* with stromal decidualization following paracrine cues.

With the field's concentration on the endometrial perivascular environment and the ESPs, several queries still need to be addressed. Whether the perivascular environment is endometrium-specific with resident progenitors and whether these progenitor cells really show *in vivo* plasticity (70). It is unclear whether the *in vitro* phenotype of ESPs (induced

multi-lineage differentiation potential) translates to *in vivo* settings. Can the ESPs, thanks to their ubiquitous distribution, contribute to tissue repair/ regeneration by replacing cells following injury?

Furthermore, the current marker combination used to isolate ESPs (CD146+ PDGFRß+) is not specific to stromal cells as these markers are also used for pericytes (mural cells and vascular smooth muscle cells)(71). It is important to distinguish between different cells in the perivascular environment in order to determine clear cell profiles and understand their specific role in regeneration. Guimarães-Camboa et al. explored the role of pericytes (mural cells and vascular smooth muscle cells) from multiple sources (heart, brain, skeletal muscle, and adipose) in an *in* vivo mouse model, showing that labeled pericytes did not differentiate or alter their cell identity and plasticity in aging or following injury. However, once expanded *in vitro* they presented an MSC phenotype (72). Thus, the pericyte/ ESP questions must also be addressed (73), whether ESPs are pericytes and *in vitro* expansion induces a misleading MSC-like phenotype (74). Equally interesting are studies on bone marrow (BM) MSCs which show that CD146+ cell surface expression is regulated by oxygen availability suggesting expression is related to perivascular location/ proximity to oxygenated blood and handling rather than cell plasticity (75). Consequently, ESPs need further characterization, specifically looking at the impact of niche environment and establishing functional assays to determine their role in regeneration.

Overall, the heavy reliance on a single stromal progenitor within the perivascular environment has limited the understanding of the greater stromal compartment. It is highly likely that a heterogeneous stromal compartment ensures tissue regeneration with complex interactions between multiple stromal populations and immune cells as seen in other tissues e.g. lung, lymph node and prostate(76-78).

1.4.2 EPITHELIAL STEM CELL NICHE

Based on gland-regulated re-epithelization following menses, EEPs are thought to originate from the *basalis functionalis* interface. Unlike ESPs, long-term expansion of epithelial cells in monolayer culture is not possible, due to senescence and loss of polarity, without the contribution of niche factors or a feeder layer of heat-inactivated fibroblasts (79). In one of the first *in vitro* studies exploring EEPs, endometrial glands were stained for sry-box

transcription factor 9 (SOX9), β-Catenin and surface stage-specific embryonic antigen-1 (SSEA-1/ CD15), all showing intense staining in the *basalis* tissue. Of the three proteins, SSEA-1 showed the highest expression in the epithelial glands. Further assays confirmed that SSEA-1 + flow cytometry sorted cells had higher telomerase activity and telomere length. The SSEA1+ fraction was also able to form larger and significantly more spheroids in Matrigel™ than the SSEA1- fraction. N-Cadherin (CHD2) has been identified as a further cell surface marker of endometrial epithelial cells with greater stem/ progenitor properties. Cells expressing N-Cadherin have been explored *in vitro* (2D clonogenicity and self-renewal assays and 3D gland-like structures), as well as through immunofluorescence in whole tissue sections looking at co-localization with Ki-67 and SSEA-1, showing these cells to be quiescent and overlapping with the SSEA-1+ population (80). Nevertheless, there haven't been any functional studies determining their role relative to other epithelial cells in the glands, in terms of hierarchy and cellular organization in the gland. Based on the characterization of the intestinal epithelial lining and the existence of adult stem cells crypts the marker leucine-rich repeat-containing g-protein coupled receptor 5 (LGR5) was proposed as a further EEP marker due to its mesodermal origin (81). *In situ* hybridization has shown LGR5 expression both in the luminal and glandular epithelium suggesting stem cells could be in both areas (82).

In the last few years, advanced organoid cultures of the endometrial epithelium have been developed using human and rodent primary cells (83, 84). These 3D cultures with more defined *in vitro* conditions including Matrigel™, different growth factors and signaling pathway mediators, can respond to hormonal cues (E2, P2, human chorionic gonadotropin, human placental lactogen and prolactin) and have long-term expansions capacity (> 6 months) without evidence of genetic instability (83). Clonally expanded epithelial organoids can be derived from a single cell at different stages of the menstrual cycle and show bi-directional differentiation capacity with ciliated and secretory subsets (83). In the pathological organoid models, organoids maintain the disease heterogeneity seen in endometriosis and endometrial cancer subtypes/ staging (85).

Organoid studies have not fully clarified the cell diversity within the epithelial glands, nor established a stem cell hierarchy comparable with that seen in the intestinal epithelial crypts yet confirm the existence of endometrial epithelial stem cells (86). For the most part endometrial epithelial organoid studies have not been able to find a conclusive marker for EEPs. Turco et al. have readdressed SSEA-1 and SOX9 expression, and observed that cells

positive for these markers did not exclusively produce spheroids. Turco et al. proposed several new signature genes for EEPs including *PROM1*, *AXIN2* and *LRIG1*, however protein confirmation in whole tissue sections couldn't be performed due to lack of antibody specificity (83).

Epithelial 3D cultures are an improvement on previous 2D cultures, however it is not certain whether the chemical conditions alone replace the stroma and immune cell niche. Self-organizing stromal and epithelial cell organoids have been grown in scaffold-free agar inserts for 14 days but do not reassemble following passaging (79). As more epithelial stem cells and epithelial cells show evidence of de-differentiation from their terminal state by modulation of their niche (87, 88), it becomes clear that the interactions of the surrounding stroma provide more than just Wnt-signaling, bone morphogenic protein (BMP), BMP inhibitors and growth factors to the environment. In the intestine, the role of the mesenchyme in maintaining the stem cells is recognized, with fibroblasts, perivascular cells, smooth muscle cells and endothelial cells contributing soluble factors but also structural components (86). Multiple populations of stromal cells have been explored in rodent models to evaluate how their ablation/ modulation affects the epithelial cell niche and Wnt-signaling. Some of these stromal subsets are: FOXL1+, GLI1+ and CD34+GP38+aSMA– cells (89-91). Research on these protective stromal populations is preliminary yet a similar stromal population may exists in the endometrium where a SM22α+CD34+KLF4+ stromal population has already been identified and seen to participate in epithelial wound healing (92). In the past efforts have existed to identify a single stem cell to explain the scarless regeneration seen in the endometrium, however translational evidence from other organs and initial studies in the endometrium suggest a more complex interplay between different cell types, their plasticity and niche.

1.5 BENIGN ENDOMETRIAL PATHOLOGIES

1.5.1 HEAVY MENSTRUAL BLEEDING

Heavy menstrual bleeding (HMB) is the most common menstrual disorder and a clear example of a pro-inflammatory disorder where the transition from controlled inflammation is impaired preventing swift coagulation and wound repair. Structural abnormalities in the uterus environment (polyps, adenomyosis and leiomyoma) can contribute to abnormal

bleeding patterns but are usually identified by ultrasound or magnetic resonance imaging. Unlike other benign gynecological disorders, unexplained HMB shows no apparent histological abnormalities, specifically with regards to glandular structures, distribution of glands or the number of arterioles in the tissue (12). This may suggest a disruption of the stromal compartment. HMB patients have significantly higher levels of TNFα protein expression in menstrual effluent than healthy women, while levels of MMP2 and MMP9 are significantly lower (93). Furthermore, HMB patients have increased collagenase and fibrinolytic activity while showing reduced expression of procoagulant factor and plasminogen activator inhibitor-1 (94, 95). Increased expression of COX1 and COX2 mRNA and PGE2 protein levels have also been observed in unexplained HMB, possibly explaining impaired clotting, stromal cell directed ECM remodeling and angiogenesis. There has also been evidence of lower hypoxia-inducible factor 1-alpha (HIFα) protein in women with HMB, as well as, its downstream targets VEGF and platelet factor 4 (96). No specific pathway has singularly been identified as responsible for HMB, thus multiple therapeutic approaches are being taken to improve clotting and limit inflammation and bleeding including: progesterone receptor modulators, synthetic androgens, non-steroidal anti-inflammatory drugs, gonadotrophin-releasing hormone agonists/ antagonists, progestins and hormonal contraceptives (12).

1.5.2 PROGESTERONE RECEPTOR MODULATOR ASSOCIATED ENDOMETRIAL CHANGES

Since the discovery of the progesterone receptor (PR) in the 1970s, agonists/ antagonists of PR and synthetic progestins have been explored as therapeutic agents for benign gynecology conditions (97). The current set of ligands are classified as progesterone receptor modulators (PRM) which include several investigational compounds and clinically approved pharmaceuticals such as mifepristone, ulipristal acetate, asoprisnil, and vilaprisane. Although the precise mechanisms of action of PRMs remain unknown and may slightly vary depending on the specific compound, PRMs bind the PR thereby modulating the actions of native P4. Likewise, some PRMs modulate the estrogen receptor e.g. ulipristal acetate (98-101), while mifepristone has been seen to also modulate the GR (98-101). PRMs are of clinical interest in medical abortion, emergency contraception and management of symptomatic uterine fibroids. Ongoing research is also investigating their use as non-E2 daily oral contraceptives,

in slow-release contraceptive devices and therapeutically to induce amenorrhea or limit endometriosis and adenomyosis(12).

Mifepristone was the first PRM developed and has a two times greater affinity to PR than P4 (102). The effect of mifepristone on the reproductive system is dose, cycle-day and frequency dependent. At a high concentration of 10 mg/day follicular development is inhibited by the systemic suppression of gonadotrophins and E2 secretion is significantly reduced (12, 103). At a lower dose of 2 mg/day ovulation is delayed without preventing follicular development with disruption to the regeneration/ differentiation of the endometrial *functionalis*. Endometrium E2 levels are comparable to those seen in the mid-proliferative phase (12, 103). Consequently, during long-term low-level use of mifepristone the endometrium is exposed to unopposed E2 levels, an environment not seen under physiological conditions.

Uterine leiomyoma or fibroids are benign tumors derived from a single myocyte in the myometrium. Although most fibroids do not present with symptoms, they can have a severe impact on quality of life. The most common symptom is HMB, followed by pelvic pressure and pain. Women can also suffer from infertility due to the fibroid's intra-cavitary location where they exerting pressure on the endometrium (104). Oral administration of PRMs reduces HMB and may also reduce fibroid size (98-100), however, long-term continuous PRM use causes specific non-physiological endometrial changes in up to 60% of treated women (100). Importantly, these changes are reversible with the termination of treatment (100). Pathologists have called these changes progesterone receptor modulator associated endometrial changes (PAEC). Histological characteristics include: extensive dilated cystic glandular formations with watery content, inactive glands with low mitotic or quiescent epithelial cells, densely packed stroma not comparable to decidualization and an altered appearance of the spiral arteries (103, 105, 106). A further concern has been that long-term exposure to PRMs may cause excessive thickening of the endometrial stroma (hyperplasia) through uninterrupted E2 exposure, resulting in a pre-malignant phenotype seen in the fibroblasts.

For the most part, pathologists have classified these changes as benign however there has only been limited molecular validation of this assessment (99, 107). For the most part, researchers have looked at the altered tissue physiology collectively (primarily by histological approaches) rather than looking at specific cellular processes or cell types. However, in one i*n vitro* study the effects of E2 and P4 are inhibited by the addition of

ulipristal acetate to cell culture, which results in inhibition of pathways controlling cytoskeletal remodeling, cell migration and tissue reorganization (108). Additionally, Wilkens et al. reported that asoprisnil down-regulates the expression of genes associated with endometrial immune cell function in the stromal compartment, specifically downregulating IL15 and decreasing the abundance of uterine NK cells. Although opinions differ on the overall effect of PRMs on the tissue, the clinical concern remains, with current models of treatment employing an interrupted schedule with 12 weeks of treatment followed by withdrawal to allow endometrial shedding (12). As the use of PRMs extends beyond induced abortion, miscarriage, fibroid treatment and emergency contraception to possible regular contraception, there is a need to ensure the drug safety and understand how prolonged use may affect a complex tissue composed of multiple cell types and niches.

1.5.3 ASHERMAN'S SYNDROME

In Asherman's syndrome (AS) scar tissue adhesions form between the uterine walls obstructing blastocyst migration and implantation. Adhesions can have a mixed composition containing endometrial, connective and muscular tissue which reveals the possibility of multiple cell compartments being affected (109). A grading system classifies the condition into degrees of severity **(**See **Table 1**) ranging from endometrium with thin adhesions in low grade AS, to extensive fibrosis of the endometrium *basalis* and myometrium with reduced vascularization in heavily affected patients. In the two most severe classifications patients experience disease reoccurrence after surgery and infertility. Importantly, due to the functional layer being non-responsive to E2 and P4 regulation, the menstrual cycle may cease to exist altogether (110). In the assisted reproductive technology setting, endometrial thickness is frequently used to determine clinical responsiveness to hormone replacement therapy. An atrophic endometrium with endometrial thickness ≤8 mm during the window of implantation may suggest impaired regeneration, presumably due to an inhibition of stromal expansion in the proliferative phase (111). Consequently, AS patients and repeated implantation failure (RIF) patients are considered for similar treatments targeting endometrial regeneration.

Frequently, AS results from surgical trauma or chronic inflammation caused by infection e.g. genital tuberculosis or irradiation (112). However, idiopathic AS cases exist too (113). On a cellular level, the pathology of AS has not been determined however we hypothesize a

deregulated inflammatory and healing response in the menstrual cycle. Disruption to the functional and basal glands may also play a role in AS (114, 115). Likewise, a perturbed perivascular environment and vascular occlusion could limiting hormonally regulated stromal proliferation and angiogenesis (115). AS endometrium presents an altered stromal composition and ECM compared to healthy endometrium with upregulated expression of the proteases disintegrin and metalloproteinase domain-containing protein (ADAM)15 and ADAM17, as well as TGFβ1, SMAD family member 3 (SMAD3) and SMAD7 (116, 117).

Fibrosis is the aberrant accumulation of myofibroblasts and ECM products leading to chronic scar formation (118). The emerging picture in fibrotic research suggests that chronic exposure to pro-inflammatory and pro-fibrotic cytokines, ECM deposition and aberrant healing may deregulate stromal cells and push them towards myofibroblast differentiation. This has been explored in numerous tissues, including skin (119) kidney (120) heart (121) and bone marrow (122). Taking this into consideration, it has been proposed that deregulation of inflammatory processes in the endometrial stroma are exacerbated in AS. The combination of initial uterine trauma and low E2 levels may contribute to fibrosis rather than replenishing the stroma with dysregulated eSCs exacerbating the condition and preventing regeneration (123, 124).

Although adhesions and damaged *functionalis* can be removed via hysteroscopy, adhesions frequently reoccur (110). The current line of treatment is repeated hysteroscopies to clear the uterine cavity of scar tissue, followed by insertion of an intrauterine device and E2 treatment. As of recently, alternative/ supplementary treatments are being tested *in vitro*, clinically trialed and discussed. These include several anti-inflammatory/ regenerative therapies including angiogenesis promoting cell therapies (125), stromal cell therapies (126, 127), intra-uterine platelet rich plasma infusion (128), hyaluronic acid gel (129), intrauterine devices (E2) or insertion of an intrauterine balloon (130).

Table 1: Intrauterine adhesions classification system issued by the European Society of Gynecological Endoscopy (adapted from Yu *et al.,* 2008)

Grade	Extent of Intrauterine adhesions
I	**Thin or flimsy adhesions** Easily ruptured by hysteroscope sheath alone Cornual areas normal
II	**Singular dense adhesion** Connecting separate areas of the uterine cavity Visualization of both tubal ostia possible Cannot be ruptured by hysteroscope sheath alone
IIa	**Occluding adhesions only in the region of the internal cervical os** Upper uterine cavity normal
III	**Multiple dense adhesions** Connecting separate areas of the uterine cavity Unilateral obliteration of ostial areas of the tubes
IV	**Extensive dense adhesions with (partial) occlusion of the uterine cavity** Both tubal ostial areas (partially) occluded
Va	**Extensive endometrial scarring and fibrosis in combination with grade I or grade II adhesions** With amenorrhea or pronounced hypomenorrhea
Vb	**Extensive endometrial scarring and fibrosis** In combination with grade III or grade IV adhesions with amenorrhea

1.6 MESENCHYMAL STROMAL CELLS AND CELL THERAPY DEVELOPMENT

1.6.1 MESENCHYMAL STROMAL CELLS

MSCs were first reported in 1968 with the discovery by Friedenstein et al. that bone marrow aspirate contained fibroblast-like cells which could adhere to plastic, form colonies and differentiate into osteoblasts *in vitro (131)*. However, since their discovery, cells fulfilling these basic criteria have been derived from numerous stromal compartments including adipose (132), umbilical cord (133) and placenta (134).

As the number of MSC sources has grown, it has become clear that the initial characterization criteria apply to a heterogeneous cell population. Consequently, in 2005 the ISCT suggested nomenclature changes, advising to exchange the term mesenchymal stem cell for mesenchymal stromal cell (135). Amongst the different MSC sources, only a proportion of cells present self-renewal and multi-lineage differentiation potential as would be expected of a stem cell. Furthermore, the ISCT set criteria regarding MSC phenotype and function, defining an MSC as a cell, which adheres to plastic when cultured *in vitro* and upon induction can differentiate into adipocytes, osteoblasts and chondrocytes. Moreover, they have positive cell surface expression of CD73, CD90 and CD105 (> 95%), and are negative for the hematopoietic markers CD34, CD45, CD11, CD14, CD19 and HLA class II (< 2%). Changes in MSC guidelines have addressed some of the misunderstandings of the rapidly expanding field, however many remain. BM MSCs account for up to 0.01% of mononuclear cells. Thus, BM MSCs represent a rare fraction of cells, which are primarily studied *in vitro* following expansion, while less is known about their role *in vivo* (136). Additionally, in the rush to align different stromal cell sources with MSC criteria there is less focus on the molecular differences between these MSC populations. These molecular differences are presumably specific to a particular niche, function and organ, and thus warrant investigation.

Clinical trial results suggest that MSCs participate in wound repair and tissue homeostasis by suppressing ongoing inflammatory processes and supporting endogenous repair responses, rather than directly replacing damaged tissue (137). Thus, researchers have focused on characterizing MSC immunomodulation, distinguishing between MSCs' ability to sense their inflammatory environment and the effect MSCs have on specific immune cells through direct contact or their secretome.

1.6.2 MESENCHYMAL STROMAL CELL IMMUNOMODULATION

MSCs sense foreign DNA and pathogens through pathogen-associated molecular patterns (PAMPS) on their cell surface (138). As a consequence, they mediate chemokine and cytokine cascades initiating the inflammatory response and leukocyte recruitment in infection and wound healing. By extension, MSCs have the ability to respond to their inflammatory environment, changing their phenotype accordingly and secreting a battery of mediators specific to the milieu. It has been hypothesized that MSCs can be both "activated"

and “resting” (139) (See **Figure 4** for “activated” MSC immunomodulation). At “resting” state, MSC co-exist with leukocytes in the microenvironment conserving T and B cell quiescence and survival. They protect T cells from activation-induced death by down-regulating fas cell surface death (FAS) receptors on their cell surface, as well as, the corresponding FAS ligand on T cells (140, 141).

1.6.2.1 KEY SOLUBLE FACTORS

When MSCs are exposed to pro-inflammatory cytokine licensing via IFNγ and tumor TNFα, MSCs shift to an immune-suppressive, “activated” phenotype secreting anti-inflammatory mediators including PGE2, IL6 and indoleamine 2,3-dioxygenase (IDO) (139, 142-144). These soluble factors are part of a greater feedback loop signaling to immune cells affecting their recruitment, proliferation, activation and differentiation(33).

IDO is a catabolic enzyme that converts tryptophan to kynurenine. Increased secretion of IDO from MSCs accelerates the breakdown of tryptophan, reducing T cell proliferation as tryptophan is required for protein synthesis in cell metabolism, additionally IDO secretion has an anti-bacterial effect (145). MSCs constitutively secrete high levels of IL6, which upon co-culture with peripheral blood mononuclear cells (PBMCs) or exposure to pro-inflammatory cytokines, is significantly elevated (146). Djouad et al. reported that IL6 levels correlated with the number of MSCs in culture and inversely correlated with IFNγ and TNFα detected in culture media (146, 147). IL6 has been shown to reverse dendritic cell maturation and inhibit T cell activation while inducing monocytes towards M2 differentiation (147). IL6 production has also been linked to PGE2 secretion, with PGE2 inhibition reducing IL6 secretion (147). Aggarwall et al. demonstrated that inhibition of PGE2 synthesis mitigated the immunosuppressive effects of MSCs in co-culture with PBMCs, increasing T and dendritic cells proliferation(143).

MSCs also regulate their cell surface expression of HLA class I and II. At resting state MSCs have low HLA class I expression and low/ absent expression of HLA class II, however following IFNγ licensing MSCs upregulate HLA class I expression and induce HLA class II expression (62). With regards to HLA class II expression, differences have been observed between MSC sources. While seven days of IFNγ licensing was required for full surface expression of HLA class II in adult BM MSCs, only two days were needed for adult

MSCs(148). In other words, depending on the stromal source and developmental stage, HLA regulation can vary.

Recently, MSC derived extravascular vesicles (eVs) including exosomes and micro-vesicles have been explored for their role in immunomodulation. eVs can be released from cells and contribute to intra-cellular communication by means of transferring protein, lipids and RNAs(149). eVs have also been shown to express TLR4 enabling them to respond to exogenous and endogenous signals (149). Much like MSCs, in an *in vitro* setting, eVs can inhibit T cell differentiation and induce Tregs while reducing IFNγ production (150, 151). eVs can also contribute to the induction of a M2 phenotype in monocytes and reduce levels of pro-inflammatory cytokines (IL1β, TNFα) (152).

1.6.2.2 COMPLEMENT

Complement is a part of the early innate immune response, and has been associated with antibody mediated transplant rejection and the removal of MSC from circulation in the context of the Instant Blood Mediated Inflammatory Reaction (IBMIR)(153). Complement activation can occur via three routes (classical, lectin or alternative). Each route results in multiple cleavage steps of complement component 3 (C3) into C3a and C3b and subsequent cleavage of C3b into iC3b and C3dg. These products are bound to the MSC surface and act as ligands recruiting leukocytes. Complement activation reduces infection by increasing the recruitment and activation of phagocytes, and can induce cell injury by the membrane attack complex (MAC) which disrupts the cell membrane(33). In clinical trials, higher levels of complement anaphylatoxins C3a were found in patients treated with an intravenous (IV) infusions of MSCs indicative of complement activation (154). Consequently, it has been hypothesized that in binding complement fragments to the cell surface of MSCs, MSCs may control and mediate leukocyte recruitment and their immunophenotype (155-157). This would explain, in part, how MSCs with bound C3 may indirectly suppress PBMC proliferation (158). Additionally, the binding of complement anaphylatoxins C3a and C3b via their receptors to the MSC surface has been shown to reduce the effect of oxidative stress on MSCs and enhance their anti-apoptotic mechanisms (159). On the other hand, MSCs have been shown to express the complement inhibitors CD46, CD55 and CD59 on their cell surface, enabling them to partially limit activation (33).

1.6.2.3 MESENCHYMAL STROMAL CELL MODULATION OF T CELLS

An imbalance between the different effector T cells and Tregs contributes to autoimmune and inflammatory diseases, as well as to transplant rejection, making them important leukocytes to study with regards to MSC immunomodulation. Activated T cell proliferation is inhibited by MSC co-culture, affecting both CD4+ and CD8+ T cells (160-162). MSC-mediated T cell inhibition has been associated with down-regulation of activation markers CD25, CD38, and CD69 on the surface of both CD4+ and CD8+ T cells (163). Furthermore, Groh et al. found that monocytes were required in the co-culture for MSC-induced CD4+ T cell suppression; this dependency was not seen when other lymphocytes were depleted e.g. B cells (160, 163). Several soluble factors have been associated with T cell proliferation inhibition by MSCs, with most studies agreeing on the roles of TGFβ1, PGE2 and IDO (161, 162, 164-166). While IDO-mediated T-cell inhibition is MSC activation dependent, PGE2 and TGFβ1 are constitutively secreted by MSCs, although further up-regulated by IFNγ and TNFα licensing (164, 165). Several studies have demonstrated that T cell proliferation inhibition is dose dependent, meaning relative to the number of MSCs in the co-culture and their secretory profile (162, 167). MSC co-culture induces cell cycle arrest at G0/G1 in the proliferation inhibited T cells but does not induce cell death, with similar effects on cell cycle seen in B cell MSC co-culture (160, 168, 169). Overall, these series of experiments suggested that MSCs of different origin can suppress T cell proliferation in a dose-dependent manner, with monocytes indirectly mediating the suppression through soluble factors.

A handful of studies have studied the effect of MSCs on memory T cell differentiation. Krampera et al. demonstrated that MSCs inhibit both naïve and memory (antigen-experienced) T cell responses to their cognate antigens, affecting cell proliferation, cytotoxicity (percentage of IFNγ producing CD8+ T cells was lower) and the number of target antigen-reactive T cells (170). In another study with MSCs derived from BM, placenta decidua and amniotic fluid, prior to co-culture the number of memory T cells was higher than the number of naïve T cells, however following the co-culture the proportions were reversed with naïve T cells significantly increased while the memory T cells decreased (161). Although there has been conflicting data concerning the effect of MSC co-culture on naïve T cells, several BM MSC studies demonstrate suppression of memory CD4+ T cell differentiation suggesting an overall effect of a more immune tolerant profile (166, 171).

MSC IL10 induction has been suggested to limit memory T cell differentiation specifically within the CD 4+ T cells (166).

Tregs contribute to the maintenance of self-tolerance and regulation of autoreactive immune cells (172). Additionally, the frequency of circulating Tregs is considered a diagnostic and prognostic marker of outcome in transplantation research and Graft-versus-Host-Disease (GvHD) (173). Therefore, their induction from CD4+ T cells and CD8+ T cells, and proliferation has been studied in co-cultures with human MSCs (146, 161, 164, 174-176). MSCs in co-culture with PBMCs can induce the formation of Tregs (CD4+CD25HighFoxP3+) through cell to cell contact and soluble factors (146, 164, 174-176). This is partially controlled through MSC secretory factors such as TGFβ1 and PGE2, however also by the indirect actions of monocytes and their secretory factors including CCL18 (146, 164).

1.6.2.4 MESENCHYMAL STROMAL CELL MODULATION OF PERIPHERAL MONOCYTE SUBSETS

MSCs promote the migration of monocyte progenies from the bone marrow to the inflamed tissue through the secretion of the chemokine (C-C motif) ligands, including monocyte chemoattractant protein-1 (CCL2/MCP-1), CCL3 and CCL12 (177). Monocytes respond to MSC derived IDO enzymatic activity by altering their phenotype to a IL10 secreting CD206+ M2-like macrophages (178). Additionally, anti-inflammatory M2 markers CD206, CD163 and CD80 are upregulated on the cell surface of monocytes in MSC monocyte co-culture, suggesting a skewing of their phenotype (146). One of the soluble factors controlling monocyte phenotype is macrophage colony-stimulating factor (M-CSF), which is involved in the skewing of monocytes towards M2 macrophages (146). Similarly, IL6 is an important mediator in skewing monocytes to macrophage differentiation(179) At a gene level, MSC conditioned media stimulation of macrophages results in upregulation of genes associated with the M2 macrophage phenotype including *CD163*, *GAS6*, *CCL18*, *PLTP* (146). Interestingly, in mixed activated PBMC MSC co-cultures, removal of monocytes significantly reduces the overall immune suppressive effect of MSCs (146).

MSCs can also directly modulate tissue resident macrophages skewing them from an M1 towards M2 phenotype, with increased expression of CD206, CD163 and down-regulation

of CD86 so as to suppress local inflammation and support swift wound healing (146, 178, 180-182). In MSC-macrophage co-cultures, activated MSCs and MSC-conditioned media reduces pro-inflammatory TNFα levels and cell surface expression of HLA class II, while increasing anti-inflammatory IL10. High levels of IL10 suppress neutrophil recruitment to the site of injury and effector T cell stimulation, thereby limiting oxidative damage and encouraging wound healing (183). Macrophage proliferation and migration is enhanced by MSC and MSC conditioned media indicating their role in wound clearance and repair. In mice, MSC derived PGE2 has also been shown to elevate IL10 macrophage secretion by binding to the EP2 and EP4 PG receptors on the macrophage cell surface (180).

MSC- macrophage co-culture has been accompanied with increased phagocytic activity of macrophages, an adaptation important for infection clearance and resolution of inflammation (184, 185). Furthermore, in the context of IV infusion of MSCs, MSCs are suggested to be engulfed by phagocytic cells, triggering increased cell surface expression of CD163 and CD206 on monocytes, in line with an M2 phenotype (186). During engulfment, the MSCs appear to be regulating the immunophenotype as seen by increased IL10 and TGFβ1 expression and reduced TNFα (187). Consequently, it has been hypothesized by Hoogduijn et al. that MSCs can even regulate monocytes once engulfed, altering the first responder's immunophenotype at the site of inflammation (188).

1.6.2.5 MESENCHYMAL STROMAL CELL MODULATION OF MYELOID DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSC) are a immunosuppressive heterogenous group of immune cells from the myeloid lineage, although there is limited research on their interactions with MSC, a few studies suggest they have anti-inflammatory functions (33). MSCs derived growth regulated oncogene (GRO) chemokines stimulate a tolerogenic MDSC phenotype through the increased secretion of IL10 and IL4(189). Yen et al., demonstrated that MSC derived-hepatocyte growth factor (HGF) induces MDSC proliferation, which in turn contributed to CD4+ T cell proliferation suppression *in vitro* (190). HGF has previously been linked to monocyte priming and T cell proliferation inhibition as well (191).

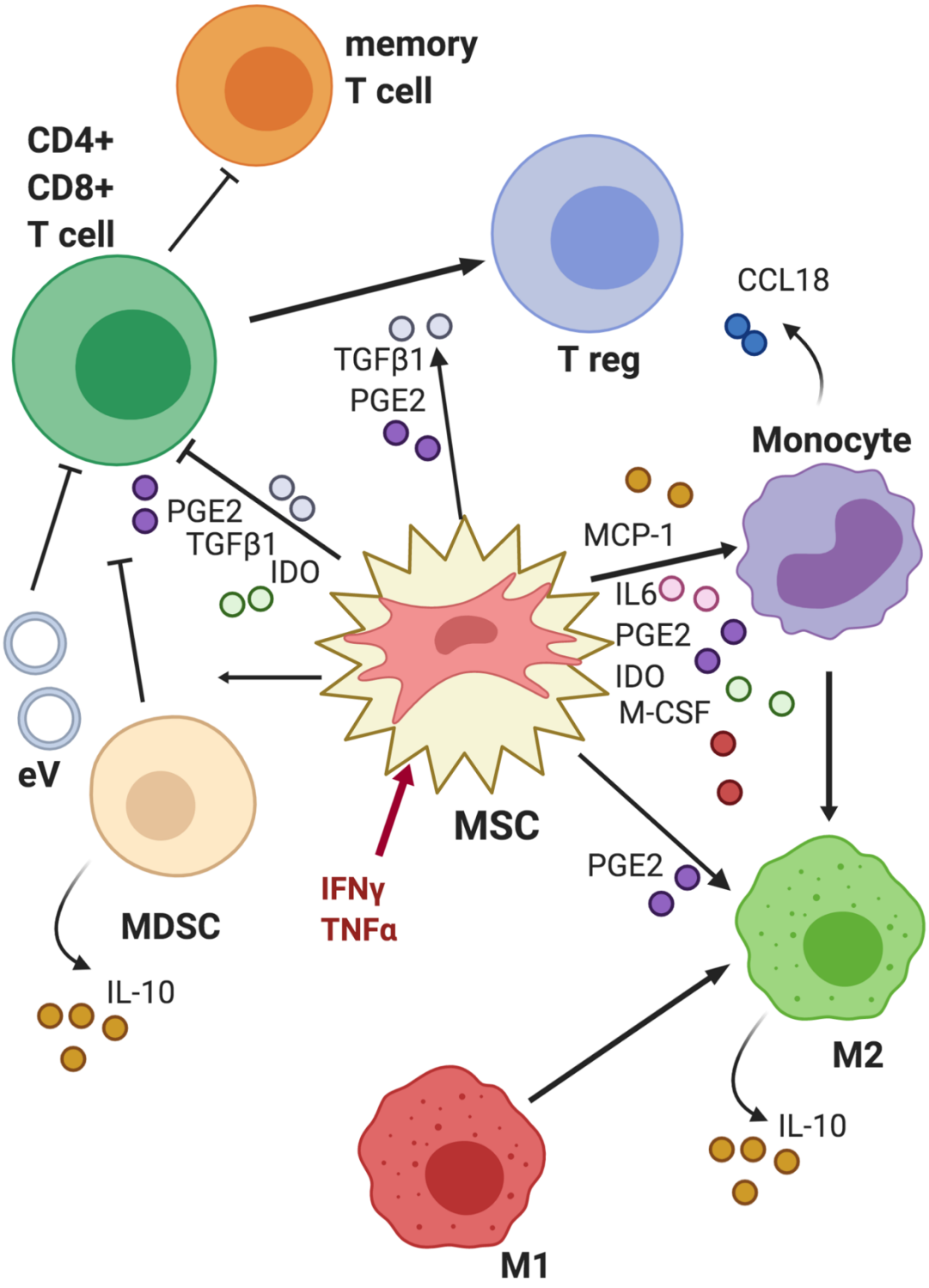

Figure 4: Mesenchymal stromal cell anti-inflammatory immunomodulation of immune cells

Activated mesenchymal stromal cells (MSC) secrete soluble factors and extracellular vesicles (eV) which regulate immune cells: effector T cells (CD4+ and CD8+), memory T cells, regulatory T cells (Treg), myeloid derived suppressor cells (MDSC), monocytes and anti-inflammatory macrophages (M2). Indirectly, MSCs induce immune cells to alter their phenotype and secrete further soluble factors. Abbreviations: (PGE2) prostaglandin E 2, (TGFβ1) transforming growth factor beta 1, (IDO) indoleamine 2,3-dioxygenase, (IL10) interleukin 10, (IL6) interleukin 6, (M-CSF) macrophage colony-stimulating factor, (MCP-1) monocyte chemoattractant protein-1, (CCL18) CC-chemokine ligand 18, (TNFα) tumor necrosis factor alpha, (IFNγ) interferon gamma, (M1) macrophage subtype 1. The diagram was created with BioRender.

1.6.3 MESENCHYMAL STROMAL CELL THERAPY

MSCs are investigated as a cell therapeutic due to their immunomodulatory capacity, with 232 clinical studies registered as active or completed on clinicaltrials.gov (May 7th, 2020), involving the search term "mesenchymal stromal cell". MSCs were first applied as a cell therapy to support hematological recovery in the adverse setting of acute GvHD in one patient in 2004, and later in a multi-center phase II clinical trial including 55 patients, reported in 2008 (192, 193). After the preliminary success of MSC therapy with GvHD, MSC therapy was trialed in other inflammatory diseases and autoimmune diseases e.g. Crohn's disease (194), arthritis (195) and lupus (196). However today, clinical trials also exist delivering MSCs as a regenerative therapy in the context of fibrosis and ischemia as seen in ischemic heart disease, liver cirrhosis, lung disease and AS (112, 197).

Although originally engraftment and proliferation of MSCs was the desirable and expected effect, more recent studies suggest that MSCs are cleared within 24 hours of intravenous (IV) infusion (198-201). Yet despite brief survival of MSCs in the circulation, their therapeutic effectiveness remains and they are able to modulate the host's immune response (193, 202). As a consequence, it is very important to determine the mode of action (MOA) of MSC therapy following IV infusion and by extension understand the relationship between MOA and delivery route.

1.6.3.1 DELIVERY AND SAFETY

MSCs have been administered by several delivery routes including local delivery by topical application or direct-tissue injection. The most common delivery route is IV infusion(203). Depending on the delivery route cells may have different MOAs, affected by their own viability, direct cell-contact or secretome, but equally by their interactions with blood, biomaterials and the surrounding milieu, including oxygen content. In this section I will focus on systemic infusion in line with our research on BM MSCs.

In patients, a few hours after MSC IV infusion, the majority of cells are cleared from circulation, with few or none detected after 24h hours (201). Infused cells have been reported to accumulate in the lung, with a portion also moving to the spleen and liver (198-201). An anti-inflammatory/ tolerogenic response prevails after MSCs have been cleared, an effect

suggested to be due to MSCs' ability to interact with the innate immune compartment of peripheral blood. Several mechanisms have been proposed based on *in vitro* human and *in vivo* rodent models, suggesting direct and indirect MOAs. De Witte et al. have demonstrated MSCs are phagocytosed by monocytes, skewing them towards an anti-inflammatory phenotype (203). Indirectly, MSCs may immunomodulate through the release of paracrine factors within eVs or by the fusion of MSC microparticles with the cell membrane of pro-inflammatory CD14+CD16+ monocytes, inducing selective apoptosis (204). Even if MSC are cleared by the innate immune response, it is still important that they are viable upon infusion in order to exert a therapeutic effect, with heat inactivated cells not eliciting the same response (205). Complement recognition through C3c and C5 binding to the MSC surface, may increase phagocytosis, as well, as the viability of the MSCs post-infusion (33).

A concern with systemic delivery has been blood compatibility with the possible risk of thrombosis and emboli occurrence (206). IV delivery targets the complement activation/ coagulation cascade, adding to the MSCs propensity for clot formation. These responses have previously been referred to as IBMIR (207). Another factor affecting coagulation has been TF/CD142, which, depending on the *in vitro* culture conditions of MSCs (number of passages and culture components) varies (154, 208, 209). BM MSCs at a low passage, with limited *in vitro* expansion, trigger only weak IBMIR whereas long-term expansion, exposure to activated lymphocytes *in vitro*, cryo-storage and freeze-thawing increase pro-thrombotic properties and TF expression. These differences also translate to clinical effects with increased blood activation markers. However, the hyperfibrinolysis marker D-dimer is not seen upon infusion (154). Nevertheless, comparatively to other MSC sources including placenta derived decidua and fetal membrane cells, BM MSCs trigger only minimal clotting (154, 210-212).

MSC transformation caused by *ex vivo* expansion should also be considered a safety concern. This concern was originally linked to the hypothesis of engraftment and ectopic tissue formation following MSC transplantation. Several recent studies have demonstrated and discussed that the risk of such pathological transformation is low (213, 214). Nevertheless, for each MSC source and cell product, the risk needs to be determined. Current approaches determine a cell product's genetic stability by DNA sequencing and karyotyping or assessing tumorigenicity through telomerase activity and soft agar tumor formation assays (214, 215). Further safety concerns include increased susceptibility to infection due to MSC immunosuppressive effects, and the acute and chronic immunogenicity of the MSCs (213,

216). In a recent systematic review looking into MSC IV therapy outcome, these two concerns were shown not to be significantly higher in patients treated with MSCs compared to control groups (216).

1.6.3.2 CELL THERAPY CHARACTERIZATION GUIDELINES

In the context of cell therapies, the ISCT has outlined guidelines for cell characterization, stressing the importance of potency testing as a measurement of: product quality, efficacy, consistency in eliciting a specific clinical response, appropriate dosing and stability e.g. shelf-life and interactions with other products (217). To quantitatively assess the potency of a cell product, the "active substance" and the MOA need to be defined beyond physiological/ chemical parameters like cell surface marker expression, taking into consideration the clinical condition it should alleviate. In MSCs where MOA is foremost manipulation of the immune response, the ISCT has proposed that the *in vitro* IFNγ + TNFα MSC-licensing model can be used to quantify immunomodulatory potential. Furthermore, they encourage measuring IDO secretion following pro-inflammatory licensing and characterization of specific leukocyte-MSC interactions depending on the cell therapy application (218).

According to the European Medical Agency, MSCs therapies are classified as advanced therapy medicinal products (ATMP), a class of live pharmaceutical therapies including cell, gene and tissue products. New legal frameworks put in place at an EU level, and enforced through national regulatory agencies, ensure that ATMPs are tested for quality, safety and efficacy before reaching patients (218-220). Cell therapies must be produced according to good manufacturing practice (215).

More specific guidelines exist based on the cell therapy product identity, its clinical application and MOA. To do this biological characterization justice, the product needs to be tested pre-clinically *in vitro,* and where of relevance, *in vivo,* with a series of "proof-of-concept" functional assays (217). Although *in vivo* animal models remain to be used for bio-distribution and toxicity studies, the EU regulation on ATMPs "Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials" note that non-clinical models should be performed with the most pharmacologically relevant *in vitro* or *in vivo* models (221). When animal models are needed, a similar pathophysiology as seen in patients is preferable and the 3Rs (reduction,

replacement, refinement) principles should be considered, avoiding animal testing when it results in inconclusive data (221). Specifically, for immunomodulation studies, animal models can be difficult as a functioning host immune system is needed for the therapeutic effect and considerable differences are seen in human and rodent immune responses e.g human MSCs secrete IDO for immunomodulation, while murine MSCs produce nitric oxide (142). Under these circumstances, *in vitro* and *ex vivo* cell and tissue based models and *in silico* analyses are encouraged (221).

1.6.4 ENDOMETRIAL CELL THERAPY

For combating AS and atrophic endometrium, a hand full of cell therapies have reached clinical trials comprising menstrual blood derived stromal cells (menSCs), umbilical cord (UC) derived MSCs, bone marrow-derived mononuclear cells (BMMNCs) and a peripheral blood-derived mobilized mixed population (**Figure 5**) (125-127, 222, 223). For the most part, these therapies can be categorized as either angiogenesis-promoting cell therapies or stromal cell therapies (112). Although the intended MOA for these therapies differs, their common aim is to induce endometrial regeneration (a more detailed review can be seen in Queckbörner et al. (112)).

In angiogenesis-promoting therapies, a heterogeneous population of hematopoietic cells is isolated from peripheral blood or BMMNCs and enriched for cells expressing the endothelial progenitor cell markers (CD133+) (125). Previously, it was hypothesized that BMMNCs may transdifferentiate into endothelial cells and engraft at the site of injection thereby ensuring angiogenesis, however substantial evidence suggests that this isn't their MOA, with multiple animal models showing no engraftment or trans-differentiation following intra-muscular or systemic injection (224). BMMNCs may modulate their paracrine environment by secretion of growth factors (225), however a new study shows that inactivated BMMNCs also induce an anti-inflammatory response (224). Thus, it is likely that cells do not act exclusively via their secretome but rather trigger a local immune response which activates the wound healing cascade. Mechanism aside, CD133+ BMMNC therapy has shown a promising, if temporary effect with increased angiogenesis at three and six months post-treatment through an increase in the number of mature blood vessels (CD31+/alpha-smooth muscle actin+), increased endometrial thickness, resumed menstruation and a reduction in adhesions. At 19 months post-treatment five live births were reported in a study of 18 patients (125).

Clinical trials in endometrial regeneration involving stromal cell therapies are at an early stage. Autologous menSCs were isolated and *in vitro* expanded from the menstruate (CD 28 - CD 4) of seven AS patients, and then topically delivered to the uterine cavity following endometrial scratching (126). Clinical trial participants showed increased endometrial thickness and three pregnancies were achieved after one or more rounds of therapy in the cohort. However, given that severe AS patients frequently suffer from amenorrhea, it is questionable whether a sufficient amount of menstruate could be collected and expanded from these patients. Likewise, as the specific pathology of AS remains unclear, in particular how the eSCs are deregulated in this condition, it might be controversial to reintroduce the same pathological cells back to the uterus exacerbating the already inflamed state. An example of this might be endometriosis, where we see pathological migration and proliferation of stromal cells outside the uterus potentially caused by retrograde menstruation (226).

In another clinical trial 25 AS patients were treated with UC MSCs within a biodegradable scaffold (127). Here, the patients also showed increased endometrial thickness following treatment and fewer adhesions, with 10 patients becoming pregnant within the follow-up period. Much like with the angiogenesis promoting therapies, these pilot clinical trials have not specifically investigated MOA and safety. In the past, stromal cell engraftment was assumed, with more recent studies suggesting that this mechanism is unlikely with most stromal cell therapies acting via their secretome and contact-mediated immunomodulation of their environment (227). Thus, it is no surprise that in Cao et al. no engraftment of UC MSCs was observed in the endometrium after treatment (127). Furthermore, it is difficult to distinguish between the effect of the biodegradable scaffold and the cells on endometrial regeneration.

Understanding the MOA of a particular cell therapy, will likely determine what the most efficient delivery route will be i.e. IV, trans-myometrial injection or cell infusion directly into the uterine cavity. Likewise, delivery methods including scratching, biomaterials/ scaffolds and combination therapies (angiogenesis-promoting therapies combined with stromal therapies) should also be evaluated once the MOA has been identified (228). Currently all of the existing phase I endometrial cell therapy trials have understandably included very small patient cohorts and no vehicle control groups. However, the heterogeneous patient cohorts, poorly defined cell populations, different intervals of cell therapy regimes and varying follow-up periods make it difficult to compare between the studies. Nevertheless, as the

endometrial cell therapy pilots suggest promising preliminary results, further studies are underway, with more specific cell product characterization in line with the ATMP guidelines (112). Likewise, there is a need for further pre-clinical *in vitro* studies of stromal-immune cell interactions to determine the precise MOA. From a safety perspective, none of the above studies have addressed the incidence of fetal growth restriction and placenta accreta seen in AS patients (229, 230). Likewise, the thrombotic properties of endometrium derived stromal cells should be considered, as previous research on decidual stromal cells has demonstrated enhanced triggering of the coagulation cascade as a consequence of systemic administration (210).

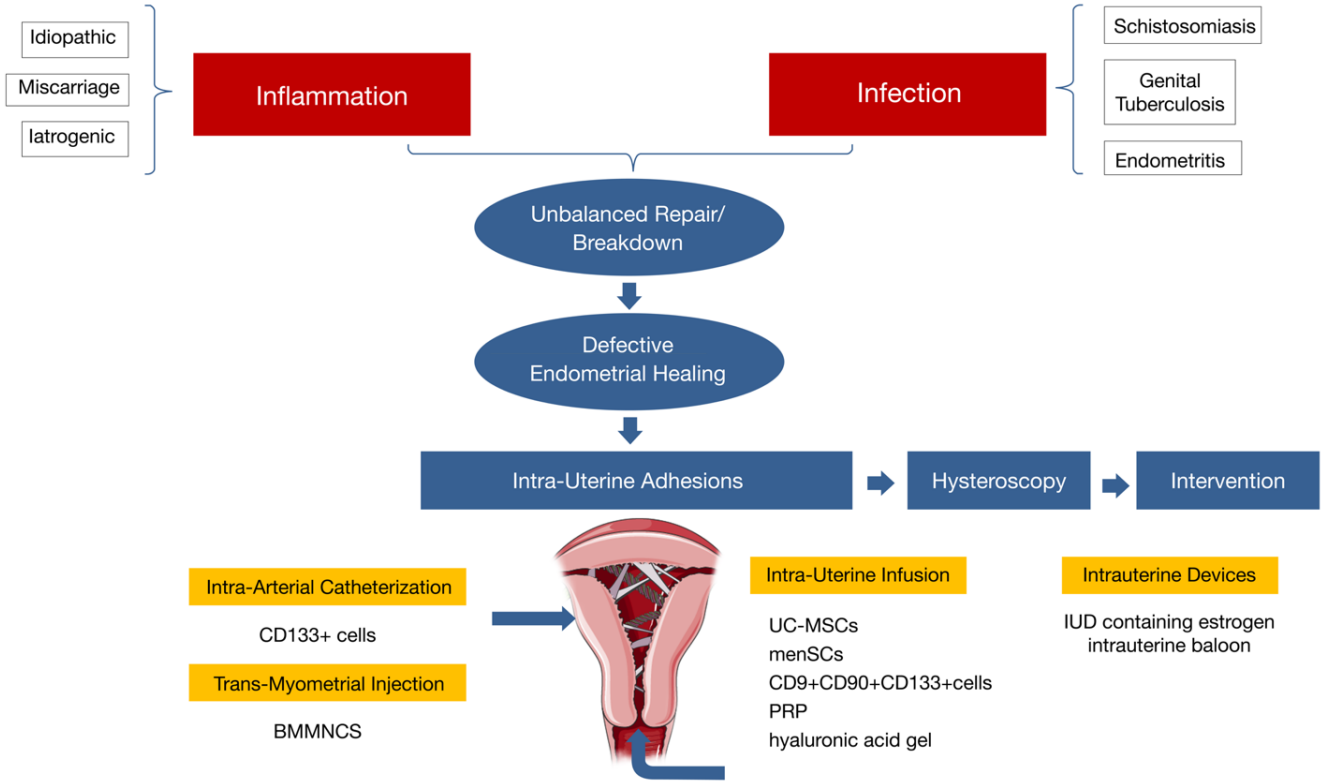

Figure 5: Endometrial cell therapies and current treatments for Asherman's syndrome

Schematic diagram of Asherman's syndrome and the current cell therapies being developed to treat it as well as existing therapies. Abbreviations: BMMNCs, bone marrow-derived mononuclear cells; UC-MSCs, umbilical cord mesenchymal stromal cells; menSCs, menstrual blood-derived stromal cells, PRP platelet rich plasma, IUD, intrauterine device. Diagram made using art from Servier Medical Art, licensed under a Creative Common Attribution 4.0 Generic.

2 AIMS

The aim of this thesis is to investigate the biological and molecular mechanisms of endometrial regeneration during the proliferative phase of the menstrual cycle. I have sought to address the equilibrium and cross-talk between hormonal regulation, immune cells and the stromal compartment.

Study I: Determine the transcriptional profile of PRM associated endometrial changes following mifepristone treatment.

Study II: Characterize the phenotype of eSCs and their immunomodulatory capacity *in vitro*.

Study III: Deconvolute the different stromal cell types, states and niches within the endometrium using single cell RNA sequencing.

Study IV: To establish the effects of blood exposure on MSC viability and immunomodulatory functions.

3 MATERIALS AND METHODS

All projects included in this thesis were approved by the Ethical Review Board in Stockholm (DNR: 02-410, 2015/367-31/4 ,2016/1582-3, 2018/1187-32).

Detailed descriptions of laboratory techniques applied in this PhD project can be found in **study I-IV** manuscripts. Therefore, only the key methods and considerations are included below.

3.1 MATERIALS

3.1.1 ISOLATION OF HUMAN ENDOMETRIAL STROMAL CELLS, EXPANSION AND CHARACTERIZATION

For **studies II** and **III** endometrial samples were obtained during the proliferative phase of the menstrual cycle. We chose this time point because the stromal cells are involved in endometrial repair and regeneration. The donor's endometrial proliferative phase (CD 7–9) was calculated based on their previous time of menstruation, and biopsies were obtained from the functional layer of the endometrium using a pipelle aspirator.

Samples were washed with phosphate-buffered saline (PBS) and minced into 2mm^2 pieces. Depending on the study, a xeno-free (**study II**) isolation or gentle isolation (detailed below; **study III**) was performed. In **study II** we establish a xeno-free protocol for future pre-clinical cell therapy development, as some clinical studies have reported higher risk of fever related acute infusional toxicity with xenogenic material (216). In **study III** a gentle isolation was required as enzymatic tissue dissociation can alter the transcriptome and lead to unwanted cell death; factors heavily discussed in the application of single cell RNA sequencing (231).

In study **II** endometrial tissue was enzymatically digested in complete media containing Minimum Essential Medium α (MEM α) with 5% pooled human platelet-lysate and heparin (20 µl/ml heparin 1000 IE/ml) using dispase II (0.75U/ml) for 45 min at 37°C and 5% CO2. This was followed by a further digestion with 1X TrypLE ™ Express Enzyme for 10 minutes at room temperature. In **study III** minced tissue was digested in complete media containing Dulbecco's Modified Eagle's Medium (DMEM) F12 and 10% fetal bovine serum using dispase II (0.5 U/ml) at 4°C overnight. The following day the tissue was digested further with collagenase III (150U/ml) and DNase (139U/ml). Isolated cells were treated with red blood

cell lysis buffer to remove all red blood cells from the cell suspension. Red blood cells are abundant in the endometrium but do not contain RNA so they cannot be sequenced.

In **study II** cells were checked for the colony-forming unit-fibroblast (CFU-F) potential by growing cells at low seeding density in complete media for 14 days. This is a functional assay developed for MSCs to determine their abundance *in vitro* (232). MSCs can self-renew even at low seeding density while fibroblasts do not grow well under these conditions. Colonies of more than 32 cells were counted, allowing exclusion of cell types with fewer cell divisions e.g. transit amplifying cells.

To further classify the eSCs in **study II**, we determined their cell-surface expression of MSC markers by flow cytometry (62). Flow cytometry is a technique that allows you to sort and functionally analyze (e.g. viability, proliferation index) cell populations based on their florescence. Cells are stained with fluorophore-conjugated antibodies and passed through a laser beam where the emitted light is characteristic of a particular fluorophore and by extension the antigen expressed on the cell surface. Using multi-color panels with different antibodies makes it possible to distinguish between cell subpopulations and quantify their abundance relative to each other. Serial monolayer expansion of eSCs on plastic will generally remove any epithelial, endothelial and residual hematopoietic cells as the environment is not favorable to their propagation. These cells require other growth factors, a feeder layer/ basement membrane substitute and specific media(84). To validate our cell population purity and MSC phenotype, eSCs were expanded until P3-4 the passage range applied in a clinical setting(193), detached from flasks and stained with the following antibodies based on the ISCT panel: CD73, CD90, CD105, CD14, CD19, CD34, CD45 and HLA class II. We also stained with live/dead marker to ensure we were only evaluating live cells as viability will affect specificity of antibody binding and autofluorescence. Similarly, we included IgG controls for every fluorophore to identify unspecific-antibody binding.

3.1.2 ISOLATION OF HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS, EXPANSION AND CHARACTERIZATION

In **study IV** BM MSCs were isolated, expanded and characterized as previously described (154). BM mononuclear cells were separated over a Percoll gradient and expanded in 5% human platelet lysate and heparin (20 µl/ml heparin 1000 IE/ml). All *in vitro* experiments with BM MSCs used cells at low passage (P2-4) which corresponded to the passages used in clinical projects(193). Additionally, cells were freeze-thawed directly before use on the day of experiments to mimic the clinical setting. Both cryopreservation and passage number are known to affect MSC functionality and immunomodulation thus we wanted to control for these variables to ensure our data was clinically relevant (233, 234). Cells were washed after thawing to remove residual dimethyl sulfoxide which has previously been linked to infusion-related toxicity in patients(235).

3.1.3 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND PLASMA FROM WHOLE BLOOD

In **studies II** and **IV** blood products were used in different co-culture conditions. PBMCs were isolated from buffy coats as previously described (236, 237). For plasma and whole blood experiments in **study IV**, peripheral blood was drawn from healthy donors using non-heparinized blood tubes without a vacuum to limit cell activation. Lepirudin was added to the blood instead of heparin to prevent coagulation, additionally it does not affect the complement system (238). Heat inactivation (HI) of the plasma causes protein aggregation and inhibits complement. For the isolation of plasma, the blood was kept on ice until centrifugation at 2000 xg for 10 min at 4°C, this ensured controlled removal of all clotting proteins.

3.2 METHODS

3.2.1 DIFFERENTIATION ASSAYS

As part of **study II** eSC characterization, mesodermal lineage differentiation potential was assessed. eSCs were evaluated for their ability to differentiate into adipocytes and osteoblasts. This is another requirement for MSC identity *in vitro* (62). However, it must be said that *in vitro* induced differentiation capacity does not directly correlate to *in vivo* multipotency (239). Osteogenic differentiation was determined by Alizarin Red staining of calcium deposits while Oil Red O was used to stain lipids produced by adipocytes. Osteogenic differentiation can only be achieved with an initially high seeding density and a confluency above 80% (240).

3.2.2 SOFT-AGAR TUMORIGENICITY ASSAY

An important consideration in ATMP development is patient safety. In **study II** several experiments were designed to assess ectopic tissue formation, the soft-agar tumorigenicity assay is an example. One property of transformed cells is anchorage independent growth, which frequently correlates with *in vivo* tumorigenicity (241). eSCs and Ishikawa cells were cultured in semi-solid agar using the CytoSelect 96-Well Cell Transformation Assay. The Ishikawa cells constituted a positive control as an endometrial adenocarcinoma cell line. At termination the 3D cultures were lysed, stained with calcein (a florescent dye) and using a plate reader the cell solution optical density was measured. By making a standard curve of known cell densities, the relationship between optical density and culture confluency could be determined. We measured initial and terminal optical density for all the donors to determine growth within 10 days. Culture confluency was an indicator of anchorage independent growth. If optical density readings decreased after culturing this indicated cell death.

3.2.3 PRO-INFLAMMATORY CYTOKINE LICENSING

As part of updated ISCT guidelines for MSC characterization, a strong focus is on their immunomodulation (242). In **study II**, the *in vitro* licensing model with IFNγ and TNFα

imitated a pro-inflammatory milieu and provided us with information on how eSCs might respond to it. Conditioned media was collected and analyzed using enzyme-linked immunosorbent assays (ELISAs) for human IL6 and PGE2. IDO activity was determined by measurement of L-kynurenine concentration, which is a breakdown product of tryptophan. IDO and PGE2 have been identified as soluble factors modulating T cells (143, 165). IL6 is an important mediator in skewing monocytes to macrophage differentiation(179). Additionally, licensed eSCs were stained for the following markers for subsequent flow cytometry analysis: CD119 (IFNγ receptor), CD120a (TNFα receptor I), CD120b (TNFα receptor II), HLA class I and II. Changes in cell surface expression of these markers were indicative of the cell's recognition and response to the licensing. In MSCs both HLA I and HLA II are upregulated by IFNγ and TNFα licensing (243).

3.2.4 PERIPHERAL BLOOD MONONUCLEAR CELLS CO-CULTURE WITH ENDOMETRIAL STROMAL CELLS

PBMC *in vitro* co-culture models provide us with information on how MSCs might directly or indirectly regulate immune cells. These assays are a further component recommended by the ISCT for stromal cell characterization and thus have been applied for eSCs in **study II** (218). PBMCs were labelled with CellTrace CFSE Cell Proliferation Kit to determine the effect of eSCs on their proliferation. Depending on the number of proliferations the fluorescent signal is reduced, each generation of cells represents a different peak on the flow cytometry histogram. PBMCs were activated with anti-CD2, anti-CD3 and anti-CD28 beads to mimic a pro-inflammatory environment and determine how eSCs may modulate them. eSCs and the activated PBMCs were co-cultured in contact and in transwell (0.4 µm membrane) for five days to identify how eSCs mediate immune cells by direct cell membrane contact or by the diffusion of secretory factors. The PBMCs were stained with antibodies suited to characterize CD4+T cell activation and differentiation. The following cell surface markers were stained for and checked by flow cytometry: CD3, CD4, CD25, CD27, CD127 and CD45RA.

3.2.5 PERIPHERAL BLOOD MONONUCLEAR CELLS CO-CULTURE WITH MESENCHYMAL STROMAL CELLS PRE-EXPOSED TO PLASMA

In **study IV** the PBMC co-culture model described above was optimized to account for the effect of blood BM MSC contact and how this modulates monocyte interactions. These assays are examples of pre-clinical *in vitro* assays to determine MOA with a specific delivery route, namely IV infusion in line with the ATMP guidelines for more specific pre-clinical *in vitro* studies (221).

BM MSCs +/- pre-exposure to plasma were cultured with PBMCs and monocyte subsets (CD14+) isolated using the Pan Monocyte Isolation Kit. The purity was evaluated using flow cytometry (92-93% $CD14^+$), it was important to have a pure monocyte population so their specific modulation could be evaluated and distinguished from the general PBMC effect. The PBMC secretome was assessed using ELISAs against TNFα a pro-inflammatory cytokine and IL10 an anti-inflammatory cytokine to determine the effect of the MSCs on the inflammatory milieu. Monocyte migration towards the BM MSC secretome was evaluated using Boyden chambers. For this, CD14+ monocytes were loaded onto 3 μm pore sized polyethylene terephthalate membrane plate inserts. BM MSC +/- pre-exposure to plasma conditioned media was added to the bottom chamber of the Boyden chamber. Monocytes were co-cultured with the conditioned media for 3 h at 37 °C / 5% CO_2. Media was subsequently removed from the bottom of the chamber and migrated CD14+ cells were counted, their viability was assessed and they were screened for chemotactic factors including MCP-1.

Following the different co-culture conditions PBMCs were stained for monocyte and macrophage markers and checked by flow cytometry to determine the effect of the BM MSCs +/- pre-exposure to plasma on the skewing of the monocyte phenotype and their macrophage differentiation: CD206, CD163, CD14, CD16 and HLA class II.

3.2.6 MICROARRAY

A microarray is a chip with a collection of DNA probes, during an experiment sample RNA is hybridized to the chip and the signal collected from the probe provides an estimation of the gene expression in the sample. Microarray data provides comprehensive large scale gene expression profiles (14,500 well-characterized human genes) making it possible to identify dysregulated molecules and signaling pathways in a biological sample (244). In **study I** RNA from PAEC and non-PAEC samples was reverse transcribed to cDNA, further transcribed to labeled cRNA and hybridized to GeneChip® Human Transcriptome Array 2.0 ST. The raw data files of HTA 2.0 were processed using Affymetrix® Expression Console Software and expression data for all the samples were obtained with the following parameters: iterative Probe Logarithmic Intensity Error Estimation (iterPLIER) as the summarization algorithm with perfect-match GC composition-based background correction. Quantile sketch normalization was applied, and the probe sets that were not annotated were discarded from further analysis.

3.2.7 SINGLE CELL RNA SEQUENCING

Single cell RNA sequencing (scRNA-seq) is a relatively new approach to sequencing technology and provides vast knowledge on a single cell's transcriptome in a specific sample at a given timepoint. Changes in gene expression between individual cells allows biological differences to be inferred relating to cell function, activation state and differentiation trajectory. It is also used to reveal information regarding previously unknown cell types (245).

In **study III** freshly isolated, unsorted endometrial single cell suspensions were prepared and loaded on a 10x Genomics Chromium Controller instrument for single-cell gel bead-in-emulsion (GEM) formation and barcoding. The advantage of the GEM technique is that individual cells can be captured without elaborate sorting and multiple assay plates. GEM reverse transcription was performed. Once cDNA was generated, amplified by PCR and cleaned, the sequencing libraries were constructed. Three runs of scRNA-seq were performed as patient samples were collected and processed on different days according with the clinical schedule and donor availability. Each run consisted of one sample/sequencing lane with approximately 3,000 cells sequenced per sample with a sequencing depth of 50,000 reads per

sample. These parameters were considered appropriate for identification of stromal subsets within a mixed cell population. scRNA-seq output files were aligned to the human genome version 19 transcriptome using STAR mapper (246). Cell Ranger was used to process raw sequencing data.

The downstream analysis of filtered cells was primarily performed in R using the Seurat suite (247, 248). Quality control measures were performed to remove doublets and dying cells as their altered transcriptome would confound scRNA-seq results. Only cells expressing between 200 – 5,000 genes, and less than 10% of mitochondrial genes were kept. RNAs coding for mitochondrially localized proteins are upregulated in broken cells indicating loss of cytoplasmic content (249). All cells were normalized according to their cell cycle stage to ensure there was no expression bias relating to cell cycle effect within a cell type, this can overshadow differences between cell types (250). The data was corrected for batch effect using the integration tool sctransform, this ensured that differences in gene expression were not dominated by inter patient heterogeneity (251). The dimensionality of the data was reduced using principal components analysis (PCA). Elbow plot was used to select top principal components which were used for downstream Louvain clustering and visualization using t-distributed stochastic neighborhood embedding (tSNE) and uniform manifold approximation and projection (UMAP). "Reference-Based Single-Cell RNA-Seq Annotation tool SingleR was run to broadly identify cell types based on machine learning using the reference dataset "Human Primary Cell Atlas" (252). Final cell type labels were established after manual assessment using known marker genes. Velocyto was used to evaluate cell lineage by cell dynamics and RNA velocity, this is based on splicing information (253).

The main differences between the high throughput genomics techniques used in **study I** and **study III** are that whole endometrial tissue was homogenized before RNA extraction in **study I** providing pooled transcriptional information while in **study III** gene expression is captured for each individual cell. Both approaches can provide a transcriptional profile for endometrium, however the single cell approach is sensitive to differences between cell types. In a complex tissue with multiple cell types and functions, homogenized sections may disproportionally represent a certain cell type (12).

2.3 STATISTICAL ANALYSIS

In **study I**, the microarray data were tested for any interfering outliers, and quality control was assessed with MA plots which visualized any differences between the two channels (dye coupling and hybridization efficiencies). Hierarchical clusters grouped genes with similar expression patterns together and PCA provided principal gene components identifying genes that explain the experimental setting. For the downstream analysis, Significance Analysis of Microarrays (SAM) method was applied(254). Normalized data from microarray and real-time PCR were subjected to unpaired two-tailed Student's T test to find significance between groups. A p-value < 0.05 was considered significant.

In **study II** comparisons in the groups were analyzed using one-way ANOVA with paired two-tailed T test. Mann Whitney U test or Wilcoxon test was used where data was non-parametric. Normality was determined by the Shapiro-Wilk test. Equal variance was determined by Bartlett's Test and F Test when assuming Gaussian distribution. Statistical significance was assumed at $p < 0.05$).

In **study III** Changes in scaled gene expression between cell types were calculated using the MAST test (255). MAST is an improvement over the generic non-parametric Wilcoxon text. MAST uses a generalized linear model framework that considers the bimodal nature of single-cell expression data due to stochastic drop-outs. MAST offers a differential gene expression test that is custom tailored for single-cell count data. A log fold change of 2 or 1.5 and adjusted p-value of 0.05 was applied to determine highly unique genes in different subsets. The adjusted p-value/ false discover rate was based on Bonferroni correction using all genes in the dataset.

In **study IV** data were assessed for normality using the Shapiro-Wilk test, and equal variances using the F test. Parametric data were statistically evaluated using Student's T test, with non-parametric data assessed using the Mann-Whitney test (where data was unpaired), or Wilcoxon matched.

4 RESULTS

Several benign gynecological disorders including HMB, endometriosis and AS show evidence of impaired inflammatory control, wound healing and endometrial proliferation. To understand these disorders better, greater understanding of healthy menstruation and regeneration of the endometrium is needed. These parallel processes are orchestrated by close interactions between hormonal regulation, immune cells and the stromal compartment. The following results address the different elements involved in controlling the endometrial proliferative phase.

4.1 STUDY I

Although PAEC is not considered a gynecological disorder, the endometrium has an "out of cycle" phenotype which may be at least partly due to unopposed E2 levels. PAEC presents temporarily in a number of women following continuous PRM usage. The altered morphology includes dilated cystic glands and compact non-decidualized stroma (103, 106). As PRMs including mifepristone may be developed for contraceptive use, and are already being applied as a treatment for benign gynecological disorders, it is important to further investigate these morphological changes (256). PAEC is a model of disrupted endometrial hormonal-homeostasis, providing insights into E2's role in endometrial structural organization. Consequently, endometrial biopsies from women presenting with the PAEC phenotype after three months of mifepristone treatment were collected and the tissue's transcriptional profile was assessed.

4.1.1 EFFECT OF PROGESTERONE WITHDRAWAL AND CONTINUED ESTROGEN SURGE ON THE STROMAL CELL TRANSCRIPTIONAL PROFILE

The E2 surge activates endometrial repair following menses with gland led re-epithelization and stromal proliferation (257). *In vitro* and *in vivo* studies have demonstrated that E2 regulates signaling between the epithelium and stroma, activating their simultaneous proliferation (59). *In vitro* stromal cell cultures treated with E2 and P4 show changes in ECM production demonstrating the important role of hormonal regulation in stromal compartment structural reorganization (258). This study provides specific transcriptional information on the effect of E2 surge and P4 withdrawal on the stromal compartment.

4.1.2 KEY FINDINGS

- 68 genes were differentially regulated between the PAEC and the non-PAEC samples (fold change ≥2 and an FDR value ≤0.05) (**see Table 2**).
- IPA analysis confirmed no pathways relating to cancer were significantly activated or inhibited with treatment.
- Genes which showed an altered expression could be assigned largely to three functions: changes in structural organisation, cell to cell communication and cell to ECM communication.

Table 2: Microarray analysis of genes differentially regulated with a fold change ≥2 and an FDR ≤0.05 in PAEC samples compared with non-PAEC)

Gene	Gene Name	Fold Change
CRABP2	cellular retinoic acid binding protein 2	3.4
ASPM	abnormal spindle microtubule assembly	3.2
KNL1	kinetochore scaffold 1	3.1
MKI67	marker of proliferation Ki-67	3
DIAPH3	diaphanous related formin 3	2.9
ANLN	anillin actin binding protein	2.9
S1PR3	sphingosine-1-phosphate receptor 3	2.8
FBN2	fibrillin 2	2.8
SFRP1	secreted frizzled related protein 1	2.8
TPX2	TPX2, microtubule nucleation factor	2.8
MELK	maternal embryonic leucine zipper kinase	2.8
TTK	TTK protein kinase	2.8
WIF1	WNT inhibitory factor 1	2.7
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	2.7
PCDHB7	protocadherin beta 7	2.7
THY1	Thy-1 cell surface antigen	2.7
NUF2	NUF2, NDC80 kinetochore complex component	2.7
HMCN1	hemicentin 1	2.6
CENPU	centromere protein U	2.6
ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif 16	2.6
BRIP1	BRCA1 interacting protein C-terminal helicase 1	2.5
TNC	tenascin C	2.5
LOXL1	lysyl oxidase like 1	2.5
KIF11	kinesin family member 11	2.5
EDNRB	endothelin receptor type B	2.5
SNORD16	small nucleolar RNA, C/D box 16	2.4
GBP3	guanylate binding protein 3	2.4
VWF	von Willebrand factor	2.4
PRSS23	protease, serine 23	2.4
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	2.4
CD248	CD248 molecule	2.4
LOX	lysyl oxidase	2.4
PLAT	plasminogen activator, tissue type	2.4

MAP1B	microtubule associated protein 1B	2.4
PBK	PDZ binding kinase	2.3
CD34	CD34 molecule	2.2
BGN	biglycan	2.2
PDPN	podoplanin	2.2
SYT11	synaptotagmin 11	2.2
FBLN5	fibulin 5	2.2
TPTEP1	transmembrane phosphatase with tensin homology pseudogene 1	2.2
IFI44	interferon induced protein 44	2.2
MACC1	MACC1, MET transcriptional regulator	2.2
FLRT2	fibronectin leucine rich transmembrane protein 2	2.2
OAS2	2'-5'-oligoadenylate synthetase 2	2.2
DTL	denticleless E3 ubiquitin protein ligase homolog	2.1
CENPK	centromere protein K	2.1
APOE	apolipoprotein E	2.1
PXDN	peroxidasin	2.1
EPSTI1	epithelial stromal interaction 1	2.1
PRC1	protein regulator of cytokinesis 1	2.1
KIRREL	kin of IRRE like (Drosophila)	2.1
TCEAL7	transcription elongation factor A like 7	2
PAG1	phosphoprotein membrane anchor with glycosphingolipid microdomains 1	2
RNU5A-8P	RNA, U5A small nuclear 8, pseudogene	2
FAM43A	family with sequence similarity 43 member A	2
CDH5	cadherin 5	2
CHN1	chimerin 1	2
RAMP2	receptor activity modifying protein 2	2
RNU5D-1	RNA, U5D small nuclear 1	2
ITGA5	integrin subunit alpha 5	2
ADAM12	ADAM metallopeptidase domain 12	2
TRAM2	translocation associated membrane protein 2	2
ST6GAL2	ST6 beta-galactoside alpha-2,6-sialyltransferase 2	2
DHFR	dihydrofolate reductase	2
PCDHB11	protocadherin beta 11	2
COPZ2	coatomer protein complex subunit zeta 2	2
DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides 1	-2.5

4.1.3 DISCUSSION

Based on the microarray analysis and its technical validation via qPCR we were able to see that differences in gene expression between PAEC and non-PAEC biopsies were largely associated with an altered ECM (*ADAMTS16*, *FBN2*, *FBLN5*, *FLRT2* and *ADAM12*) and activity in the perivascular environment (*THY1*, *EDNRB*, *VWF*, *CD34*) within the stromal compartment. Between late secretory phase and early proliferative phase, changes in the interstitial ECM, basement membrane and vascular permeability are hormonally regulated to ensure controlled tissue breakdown and repair. Overall, temporary morphological changes seen in the histological assessments of the PAEC biopsies were accompanied with a transcriptional profile indicative of structural changes in the stroma. Study I introduced the diversity of stromal niches and how hormonal regulation affects structure and functionality.

A limitation of this study was extracting RNA from whole endometrial tissue, this may bias a gene profile to the most abundant cell-type in the biopsy and might explain why we see such a strong stromal gene profile. Nevertheless, by enzymatically digesting the tissue and analyzing the cell types individually we might have altered the transcriptional profile of PAEC.

4.2 STUDY II

eSCs are being developed as an anti-inflammatory/ anti-fibrotic therapeutic to combat AS and RIF (112, 126, 127). Although these pilot studies are promising, a thorough characterization of the eSCs is needed, encompassing their phenotypic, immunomodulatory properties and safety testing e.g. tumorigenicity (62, 214, 218, 242). We sought to investigate *in vitro* expanded, proliferative phase eSCs in line with the ISCT guidelines for minimal characterization of MSCs, their response to a pro-inflammatory licensing and their effect on CD4+ T cell differentiation and proliferation. Through karyotyping, telomerase related assays and soft-agar cultures expanded eSCs chromosomal stability and tumorigenicity was assessed.

4.2.1 PROLIFERATIVE PHASE ENDOMETRIAL STROMAL CELLS PRESENT UNIQUE IMMUNOMODULATORY ADAPTATIONS

Surprisingly little is known about the functionality of the proliferative phase eSCs and their response to cytokines and modulation of leukocytes in the context of regeneration. Previous research into the regenerative properties of the endometrium have frequently limited themselves to the perivascular environment with a strong focus on the role of the ESPs (CD146+ PDGFRβ+) in controlling regeneration rather than addressing the larger stromal tissue's cellular heterogeneity (9, 61, 67). Likewise, immunological assessments of the endometrium have focused on the window of implantation or the decidua following placentation (12, 20, 48, 259).

The stromal compartment in the endometrium undergoes major structural changes during menstruation and regeneration, taking cues from inflammatory mediators (cytokines, chemokines and prostaglandins) and leukocytes (20). In the context of cell therapy, previous clinical trials applying external stromal sources e.g. UC MSCs to regenerate the endometrium do not address the unique immune-privileged site that is the uterus, assuming MSC sources are interchangeable (222). Consequently, it is important to determine the immunomodulatory potential of eSCs and their similarity to other MSC sources. At the same time our findings should improve our understandings of healing and regeneration following menstruation.

4.2.2 KEY FINDINGS

- eSCs can be reliably isolated and expanded, form colonies at low seeding density and show no significant differences in growth kinetics within the first four passages (determined as the number of passages required to generate a therapeutic dose for clinical applications) or between donors.
- eSCs express cell surface markers in line with ISCT MSC guidelines; positive for CD90, CD73, CD105, and HLA class I (> 95%) and were negative for CD45, CD34, CD19, CD14, and HLA class II (>2%).
- eSCs are genetically stable following *in vitro* expansion, with low telomerase activity, no karytotypic changes, and low anchorage independent growth (tumorigenic potential).
- eSCs license to an anti-inflammatory phenotype without upregulation of HLA class II.

- ❖ eSCs modulate the proliferation, activation, and differentiation status of CD4+ T cells, skewing them toward an EM phenotype.
- ❖ eSCs prove to be more effective, *in vitro*, in their immunomodulation via their secretome (see **Figure 6** for summary of eSC immunomodulation).

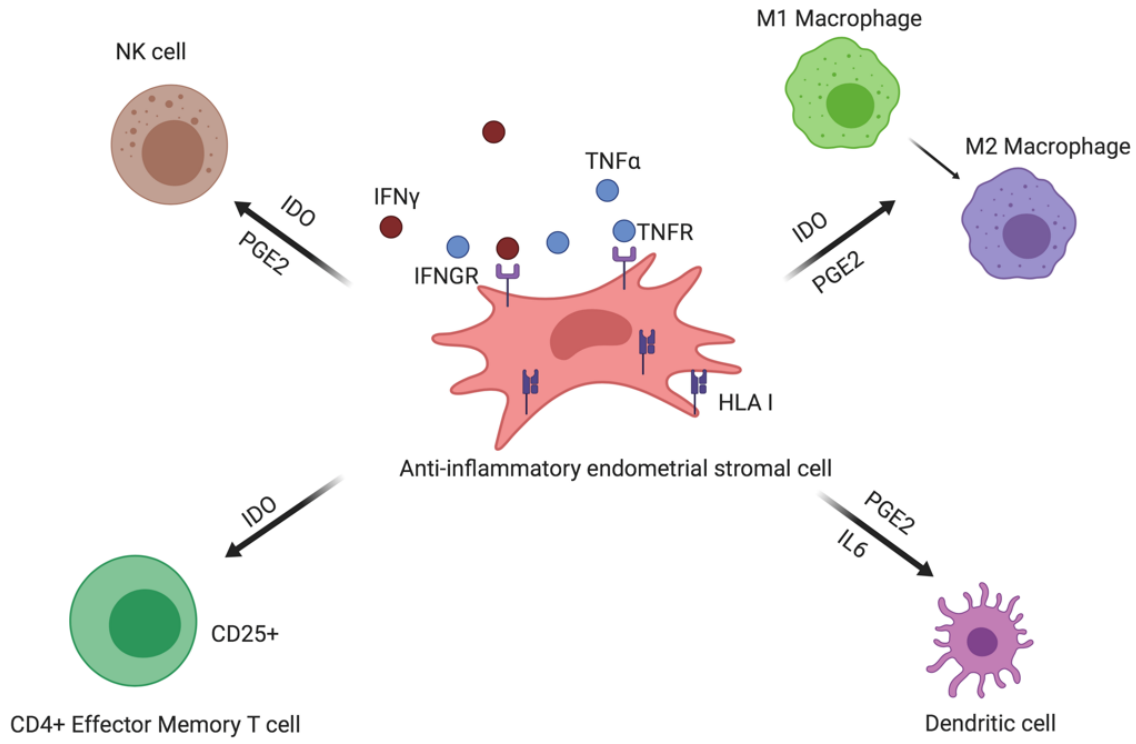

Figure 6: Endometrial stromal cell immunomodulation
Schematic diagram of endometrial stromal cells and their putative modulation of immune cells based on our pro-inflammatory licensing and activated PBMC co-culture experiments. Abbreviations: (NK) natural killer, (IDO) indolamine-2,3-dioxygenase, (PGE2) prostaglandin E2 (IFNγ) interferon gamma, (TNFα) tumor necrosis factor alpha, (IFNGR)interferon gamma receptor, (TNFR)tumor necrosis factor alpha receptor, (HLA I) human leucocyte antigen I, (IL6) interleukin 6. Diagram made using BioRender.

4.2.3 DISCUSSION

As the number of MSC therapies from different stromal sources is steadily expanding, regulatory requirements for cell characterization and MOA are adjusting, revealing unique cell properties indicative of a cell source's *in vivo* niche. This certainly applies to expanded eSCs which express the phenotypic hallmarks of MSCs, however their lack of cell surface expression of HLA class II in a licensed/ pro-inflammatory environment suggest they have a unique immunomodulatory phenotype. This distinguishes them from other MSC sources e.g. bone marrow, adipose and fetal stromal tissue(237, 260, 261).

No chromosomal abnormalities were observed in the *in vitro* expanded eSCs, they showed no anchorage independent growth and telomerase activity was low. This confirms previous MSC studies which indicate no evidence of genomic instability or tumorigenicity in expanded human stromal cells (262-265). MSCs uphold tissue homeostasis by regulating inflammation and repair through their secretome (266), consequently it is important to understand how stromal cells interact with the innate and adaptive immune system. Pro-inflammatory cytokine (IFNγ and TNFα) licensing experiments upregulated IDO, IL6, and PGE2 in eSC spent media indicating a switch to an immunomodulatory/suppressive profile. Perhaps, indicative of T, NK and dendritic cell suppression (165, 180, 267). PGE2 has been reported to promote proliferation of epithelial cells and contribute to angiogenesis suggesting its role in wound healing (236, 268). In line with MSCs, our findings show that eSCs suppress activated CD4+ T cell proliferation via cell-to-cell contact and paracrine factors when co-cultured with PBMCs (162, 167, 269). eSCs primarily modulated the CD4+ memory T cell subsets, foremost increasing CD4+ T cell differentiation towards an EM T cell state. In decidualized endometrial stroma and first trimester decidua CD8+ T cells present an EM state as well (45, 52, 54). The general EM phenotype in the reproductive tract is in line with that seen in other mucosal linings, EM T cells are the first line of defense against reinfection (270).

We conclude that the endometrial stromal compartment shares phenotypic characteristics with MSCs in terms of cell surface marker expression, ability to attach to plastic, and differentiation capacity. However, their regulation of HLA class II and memory T cell subsets suggests more tissue origin specific immunomodulation. At the same time this study demonstrated that MSC markers, colony forming properties and differentiation capacity is not unique to ESPs as eSCs representing the bulk of the endometrial stromal compartment also meet these requirements. This contributed to our curiosity to start **study III** and delineate the different populations in the stromal compartment, finding better criteria to distinguish between the subsets at a molecular level.

4.3 STUDY III

Understanding the cellular interactions between fibroblasts, progenitor cells and transit amplifying cells driving regeneration will provide us with a starting point to understand how these mechanisms are derailed in chronic inflammatory disorders e.g AS and HMB. Other stromal compartments in the lung and lymph node have eloquently shown to be composed of

different subsets indicative of niche, activation status and injury response (77, 78, 271). Consequently, we have applied single cell RNA sequencing to proliferative phase endometrium *functionalis* to delineate the complexity within the stromal compartment, and the perivascular environment, and thereby understand their interactions and combined contributions to endometrial proliferation.

4.3.1 ENDOMETRIAL STROMAL CELLS ARE A HETEROGENOUS POPULATION OF CELLS WITH ADAPTATIONS FOR DIFFERENT NICHES AND ACTIVATION STAGES

Our previous studies, and the wider literature demonstrate that eSCs respond to hormonal changes by altering the ECM composition and their rate of proliferation (258). Likewise changes in the inflammatory environment prime the cells to different activation states and trigger the release of inflammatory mediators which recruit and suppress leukocytes. Decidualization and the menstrual cascade originates in the perivascular environment where a set of stromal cells reside in a niche, taking cues from the vasculature (20). Given the knowledge of these different processes, and that menstruation and regeneration occur in parallel at different foci across the endometrium, we expected to see higher complexity in the stromal tissue with subsets of stromal cells undergoing different processes to mediate regeneration.

4.3.2 KEY FINDINGS

- Multiple stromal environments exist within the endometrial *functionalis* with unique transcriptional profiles.
- Six stromal subsets have been identified, several associated with an activated fibroblast phenotype e.g. ISG15+ cells, while others possibly regulate epithelial cells and uphold their stem cell niche e.g. BMP7+ cells or stromal cells contributing to the perivascular environment e.g. ACTA2+ and THY1+ cells(see **Figure 7**).
- Within the perivascular cell fraction, transcriptional data suggest cells can be distinguished as mural cells and smooth muscle cells.
- Current ESP markers are not specific on a transcriptional level and they cannot distinguish between smooth muscle cells, mural cells and ESP. The transcriptional

profile is more specific to perivascular location than cell type distinctions (see **Figure 8**).

- A published single cell RNA sequencing dataset was used for external validation of stromal and perivascular cell subset profiles.

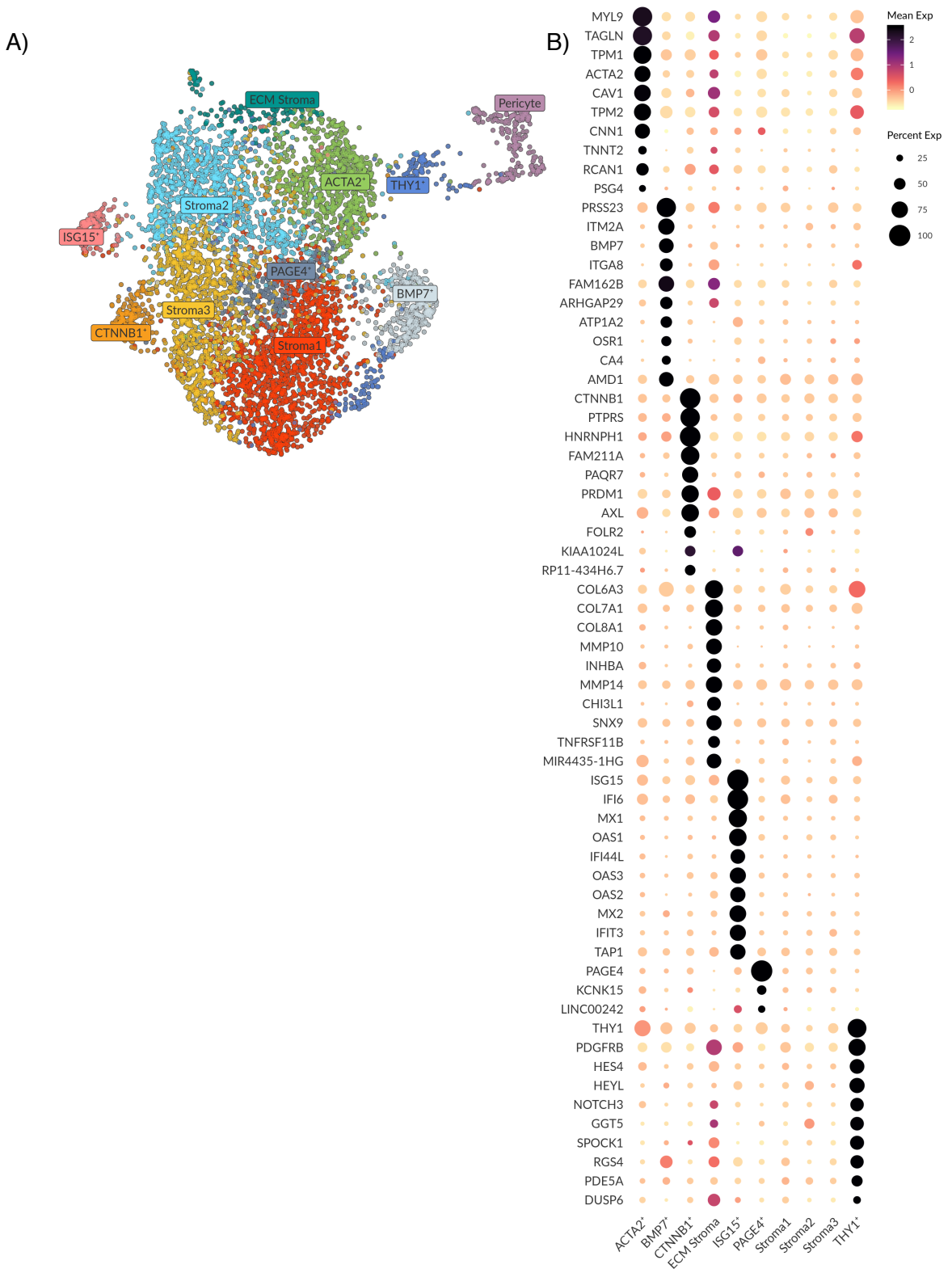

Figure 7: Analysis and subtyping of the endometrial stromal cell compartment
A. UMAP plot showing ten clusters of endometrial stromal cells and a cluster of perivascular cells on the far right. Clusters are labelled as per their identified expression profile in B. **B**. Dotplot showing the top differentially expressed genes (rows) for the ten stromal clusters (columns). Genes were selected based on MAST test with a minimum log fold change of 2 and adjusted p-value of 0.05. Note that the color scale is clipped at 2.5. Stroma 1, 2 and 3 do not show any unique expression. The PAGE4+ cluster shows a very biased differential expression for *PAGE4* only. The ECM, ACTA+ and BMP7+ clusters show a higher expression for genes involved in ECM breakdown, remodeling and organization. The CTNNB1+ cluster shows higher expression of genes involved in epithelial regulation and innate immunity. The ISG15+ cluster shows higher expression for genes involved in innate immunity. The THY1+ cluster shows higher expression of genes involved in Notch signaling.

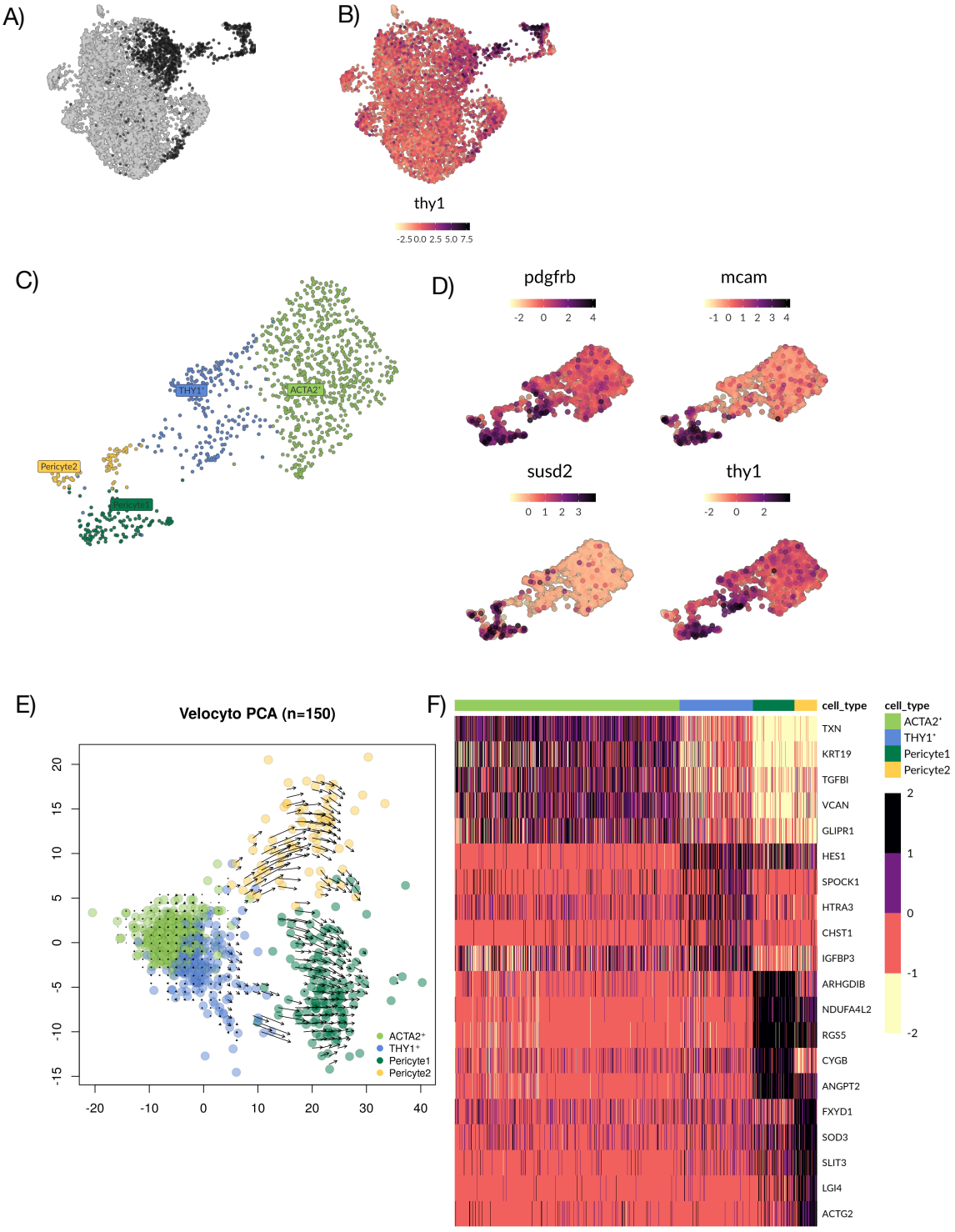

Figure 8: Analysis of cell subsets in the perivascular environment

A. UMAP plot of endometrial stromal cells and perivascular cells highlighting cell subsets ACTA2+, THY1+ and pericyte 1 and 2 (in black). **B.** UMAP plot of endometrial stromal cells and pericytes showing increased expression of *THY1* with increasing proximity to the pericyte cluster at the top right. **C**. UMAP plot showing groups ACTA2+, THY1+ and Pericyte 1 and 2. **D**. UMAP plot showing expression pattern of genes *PDGFRB*, *MCAM*, *SUSD2* and *THY1*. None of these markers can exclusively identify any of the clusters mentioned in **C**. **E**. PCA scatterplot showing RNA velocity. Predicted developmental trajectory between clusters is displayed as a vector field. Short arrows indicate a steady state and long arrows indicate active progression towards a differentiated state. Cells differentiate along the direction of the arrow, here indicating some of the THY1+ cells committing towards the Pericyte1. **F**. Heatmap showing the top differentially expressed genes (rows) for each cell cluster (columns) based on MAST test with minimum log fold change of 1.5 and adjusted p-value of 0.05.

4.3.3 DISCUSSION

Our transcriptional data suggests there are multiple stromal cell profiles which may represent different cell types, states or niches. Six profiles clearly distinguished themselves in our analysis, of which three are retained throughout the menstrual cycle and into early pregnancy (external dataset). These subsets may contribute to the tissue regeneration and warrant further protein validation and functional *in vitro* testing. Specific subsets suggest different activation states of stromal cells presumably involved in wound healing and modulation of leukocytes and cytokines. The ISG15+ population presented an interferon regulated gene profile, and based on literature and data from **study II**, this may present an activated stromal subset involved in tissue breakdown and repair (272). The CTNNB1+ population includes genes linked to M2 macrophage polarization (273-275). *In vitro* and *in vivo* studies have demonstrated that MSCs may contribute to macrophage polarization to a M2 pro-healing/ anti-inflammatory state via paracrine mechanisms (180, 276). The BMP7+ population has genes involved in myofibroblast differentiation, epithelial mesenchymal transition (EMT) and TGFβ1-Wnt signaling (*PRSS23*, *ITGA8*, *BMP7*, *ITM2A* and *ARHGAP29*) and also bear some resemblance with the intestinal mesenchyme niche, protecting and conserving the epithelial stem cells showing expression of *CD34* and *RSPO1* (86, 277-280). Furthermore, single cell RNA sequencing provides detailed transcriptional information about the perivascular environment, assumed to be the niche of ESPs (61, 67, 281). Although multiple cell types could be identified in this environment, current marker genes do not exclusively distinguish a stromal population from smooth muscle cells or mural cells in the perivascular space. It is not known whether the proteins corresponding to the mentioned marker genes are more discriminatory within the perivascular environment. Currently, perivascular cells (mural and smooth muscle cells) and ESPs are isolated and expanded using the same cell surface markers (71).Consequently, it is important to validate these findings to ensure marker specificity. Overall this study has provided descriptive data on the complexity of the stromal compartment and should act as a starting point to further investigate different stromal subsets on a protein and functional level to provide us with new knowledge to explain mechanisms controlling endometrial regeneration.

4.4. STUDY IV

As investigated in **study II**, MSC and eSCs can mediate immune cell subsets via cell-membrane mediated contact or their secretome in *in vitro* co-culture models. However, in the clinical setting MSC MOA after IV infusion is largely unknown, MSCs rarely reach the target site and are cleared from circulation rapidly (154). Nevertheless, this does not affect their efficacy and their ability to modulate the host immune system (193). When developing MSC cell therapies, MOA and potency are heavily affected by the intended delivery route. The aim of this study was to determine the effect of blood components on BM MSC viability and immunomodulation.

4.4.1 BLOOD COMPONENTS PROMOTE IMMUNOMODULATION THAT PERSISTS WITHIN THE SYSTEM BEYOND INFUSED MESENCHYMAL STROMAL CELL CLEARANCE

This study distinguishes itself from the other studies within this thesis, in its use of BM MSCs. However, the results are applicable to endometrial regeneration and the role of the stroma. In the context of endometrial cell therapy, it is important to address the delivery route early on as it will affect the MOA of the cell product, as well as their potency. MSCs have been said to trigger the instant blood mediated inflammatory response, thereby activating the complement and coagulation cascade. In the context of the endometrium, these actions are very important as we know they are both heavily involved in controlling menstruation and tissue repair (20). Additionally, placenta derived decidual stromal cells have shown a high expression of TF and thus clotting capacity, mechanisms that should be considered when designing a cell therapy and its application (154, 210).

4.4.2 KEY FINDINGS

- Exposure to blood plasma results in rapid lysis of BM MSCs with surviving BM MSCs showing reduced viability and cell swelling.
- Plasma borne C3c fragments bind to the surface of BM MSCs in a time dependent manner, modulating their expression of complement receptors (C3aR and C5aR) but not complement inhibitors (CD46, CD55 and CD59).

- BM MSCs exposed to plasma secrete reduced levels of MCP-1, resulting in the reduced recruitment of monocytes.
- MSCs exposed to plasma and whole blood modulate the phenotype of CD14+ monocytes, resulting in a predominantly anti-inflammatory CD14+CD16- classical monocytic compartment, with downregulation of pro-inflammatory CD14loCD16+ non-classical monocytes.
- The introduction of BM MSCs to whole blood increases plasma levels of IL6, modulating the phenotype of CD14+ monocytes and myeloid derived suppressor cell subsets.

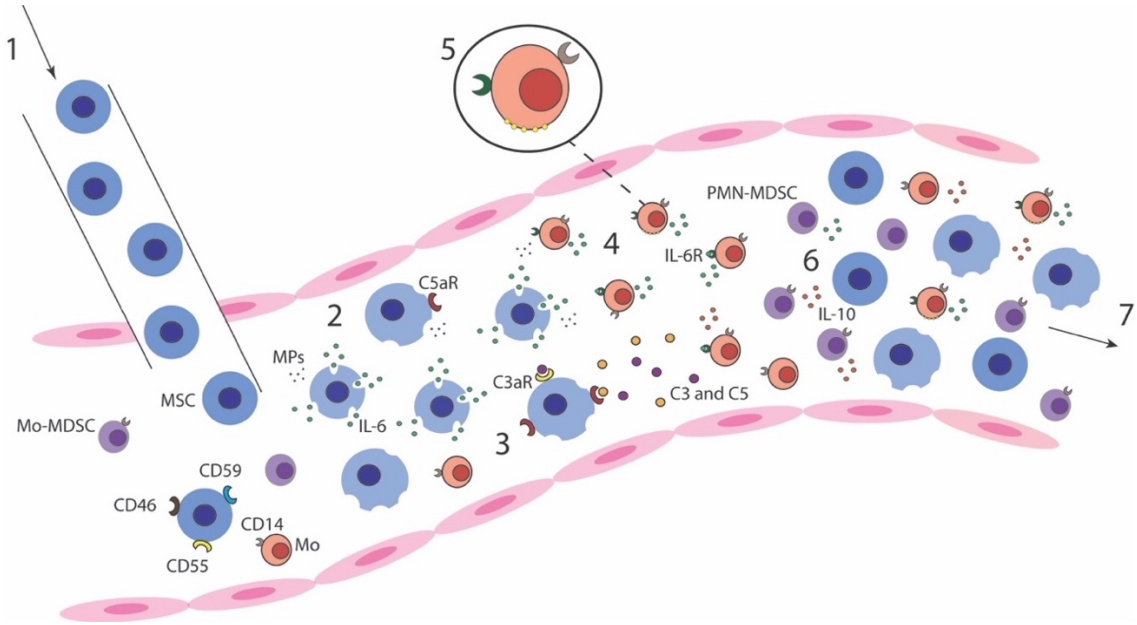

Figure 9: Following blood exposure survivor MSCs are phenotypically altered and affect maturation and functionality of innate immune cells

1. BM MSCs are intravenously infused. **2.** After exposure to blood BM MSCs' cell membrane is compromised with further mitochondrial depolarization, cell swelling and lysis. This results in a spillage of cellular contents, including interleukin (IL)-6, membrane particles (MPs) and extracellular vesicles (eVs) into the plasma. **3.** Survivor BM MSCs are subjected to complement fragments, with cell surface binding of C3c. The BM MSCs try to compensate with expression of the complement inhibitors CD46, CD55 and CD59 and by modulating their complement receptors, with downregulation of C3aR and upregulation of C5aR. **4.** The innate immune system responds with elevated IL6 levels and **5.** free MPs fuse with the monocyte cell membrane, skewing their distribution to a predominantly anti-inflammatory CD14+CD16- classical monocytic compartment **6.** There is an increase in the frequency of both monocytic myeloid derived suppressor cells (Mo-MDSCs) and polymononuclear myeloid derived suppressor cells (PMN-MDSCs). **6.** Anti-inflammatory cytokines such as IL10 and a downregulation in pro-inflammatory cytokines tumor necrosis factor alpha (TNFa) create an anti-inflammatory milieu able to skew the adaptive T cells to a regulatory profile. **7.** Remaining MSCs surviving their peripheral interactions are trafficked to the lungs, where they are cleared from the system.

4.4.3 DISCUSSION

Based on our data and the literature, BM MSCs exposed to whole blood intravenously are lost. This process may be mediated through bioactive molecules such as complement fragments. In our results, this was exemplified by our active plasma experiments where C3c fragments bound to the cell surface of the BM MSCs and increased with time. These effects were not seen in the heat inactivated (HI) plasma experiments. Significant increases in the cell area of the BM MSCs were seen in both the active plasma and HI plasma treated samples, indicating early stage necrosis as the cell swells prior to rupture. Previous research has suggested that BM MSCs modulate the innate immune response by rupturing their cell membrane and spilling out their intracellular contents (186). These things considered, there may be several cascades and mediators which induce damaging effects upon contact with blood components. The ability of the BM MSCs to modulate the innate immune system despite cell death, suggests they release free factors, membrane particles and extracellular vesicles able to trigger immunomodulatory effects by themselves. Although the cell fate of BM MSCs appears to be cell death on delivery several studies have stressed the importance of viability at the point of administration. This may be because the cells need to be alive long enough to release stored immunomodulatory factors upon delivery or give them the opportunity to modulate their secretome towards an anti-inflammatory profile. However, it would also be interesting to see how heat inactivated BM MSCs might modulate the innate immune system without their soluble repertoire but only through cell membrane contact, this has been shown to be effective in other cell therapies when determining the mechanism of action (187, 224).

General exposure to plasma demonstrated a decrease in the secretion of the monocyte recruitment chemokine, MCP-1, which led to a suppression in the directed recruitment of monocytes in culture. These data demonstrate that those cells surviving plasma contact, are able to significantly modulate their secretome within 24 hours. Furthermore, regardless of co-culture conditions (whole blood or plasma) BM MSCs significantly reduced the frequency of CD14+ monocytes, showing a particularly suppressive effect on the non-classical monocytes. BM MSCs increase the frequency of monocytic myeloid derived suppressor cells (Mo-MDSCs) and polymononuclear myeloid derived suppressor cells (PMN-MDSCs). Both subsets of these cells have immunosuppressive potential, with Mo-MDSCs secreting anti-inflammatory factors such as IL10, TGFβ1 and IL6, which have positive feedback mechanisms on monocytes to further promote their skewing away from dendritic cell

differentiation and the pro-inflammatory non-classical monocyte phenotype, as well as in the promotion of regulatory T cells (146, 282, 283).

In conclusion, BM MSC contact with blood triggers an innate immune response, resulting in an anti-inflammatory skewing of the monocyte profile, with elevated levels of classical monocytes and an increase in the frequency of both Mo-MDSCs and PMN-MDSCs. This shift in the peripheral immune repertoire may provide the foundation for modulation of T and B cells, and induction of tolerogenic responses lasting longer than the BM MSCs themselves.

5 CONCLUSION

This thesis focuses on endometrial stromal regeneration, addressing the key regulatory elements including hormones, immune cells and stromal progenitor populations. All components need to be considered when understanding endometrial healing, its deregulation in benign gynecological disorders, and in novel cell therapy development for their alleviation.

The transcriptional data in **study I** suggest that P4 withdrawal and unopposed E2 surge alter the endometrial structural organization and ECM composition particularly affecting the stromal compartment in the tissue. Morphological changes seen in PAEC do not suggest a pathological proliferative disorder with no genes identified associated with cancer progression.

Study II provides a pre-clinical characterization of eSCs for future development of cell therapy. eSCs lack HLA class II cell surface expression and skew CD4+ T cell differentiation towards an EM phenotype. Thus, we have identified a unique immunomodulatory phenotype of eSCs making it clear that they should not be considered interchangeable with other MSCs.

Study III addresses the different cell populations controlling endometrial regeneration, specifically delineating stromal subsets that might mediate tissue breakdown and repair. Several stromal subsets were identified suggesting different activation states, niches and functions. Furthermore, it addresses the perivascular niche and the controversy surrounding existing markers for ESPs. In other words, whether existing markers can distinguish between mural cells, smooth muscle cells and ESPs.

Study IV demonstrates that BM MSCs undergo cell death once exposed to whole blood explaining the clinical observation of BM MSC clearance following IV infusion. Nevertheless, BM MSCs can still modulate the innate immune response through the release of soluble factors when their membrane is ruptured and these factors skew monocytes to an anti-inflammatory phenotype. These changes in the monocyte subsets and their modulation of T and B cells could explain the long-term tolerogenic effect seen after BM MSC IV infusion.

6 FUTURE DIRECTIONS

In completing this thesis project on endometrial regeneration and cell therapy development multiple research directions have emerged.

Based on the findings in **studies II**, and **IV** and the research group's interest in endometrial cell therapy, it would be fascinating to investigate the effect of whole blood on eSCs and their activation of coagulation cascades. This would address the questions concerning ideal delivery route and safety for a potential stromal cell therapy. At the same time these experiments might provide us with new knowledge relating to menstruation, where stromal cells would be in contact with open wounds and ruptures in the vasculature. Furthermore, the unique immunomodulatory properties of eSCs in regulating HLA class II cell surface expression warrant further exploration. Our initial investigation of the *CIITA* gene and the 5 promoter regions controlling its gene expression (responsible for HLA class II protein) suggested that the gene is responsive to pro-inflammatory licensing, yet a transcriptional adaptation prevents this from being expressed as HLA class II on the cell surface. Investigating these adaptations would be interesting, especially in the current wave of induced pluripotent stem cell and embryonic stem cell therapies with HLA I and HLA II knockouts (284, 285). Understanding translational inactivation and regulation at the epigenomic level would be valuable for cell therapies and transplant tolerance. By extension, we have investigated the effect of eSCs on CD4+ T cells but haven't addressed CD8+ T cells. As CD8+ T cells have been shown to be more abundant in the secretory phase endometrium and placenta derived decidua it would be interesting to determine their differentiation state in the proliferative phase, as well as investigating the interaction between HLA class II and CD8+ T cells.

The single cell RNA sequencing in **study III** warrants considerable validation. To appropriately confirm the diversity observed within the endometrial stromal compartment RNAScope is planned for technical validation of gene-profiles, as well as, to spatially locate specific niches in the tissue relative to epithelial, endothelial and perivascular cells. The gene profiles which can be validated using RNAScope will also be checked on a protein level using immunohistochemistry. Furthermore, additional bioinformatic analysis using CellPhone DB to determine the ligand-receptor interactions between different activated stromal subsets and immune cells would provide interesting data in the context of endometrial regeneration (286). To address the endometrial perivascular environment

queries, it would be possible to stain freshly isolated eSCs with all the existing ESP markers and apply fluorescence-activated cell sorting to these cells followed by new single cell RNA sequencing and functional *in vitro* studies to determine the role of different populations in this niche. As we originally intended, it would be interesting to see the single cell RNA sequencing profile of AS endometrium so we can compare endometrial regeneration in health and disease and understand the pathology of the disease on a molecular level. In doing so, our data may provide novel targets for therapeutic intervention.

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Unfortunately, due to COVID-19 there will not be an official defense dinner to celebrate my thesis but when the situation changes, there will be a party with loads of bubbly.

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